

PACAP RECEPTOR PROTEIN, METHOD FOR PREPARING  
SAID PROTEIN, AND USE THEREOF

FIELD OF THE INVENTION

5 The present invention relates to a pituitary adenylate  
cyclase activating polypeptide (hereinafter referred to  
"PACAP") receptor protein (hereinafter may be referred to  
"PACAP receptor protein") or a salt thereof which is capable  
10 of binding a PACAP, a DNA comprising a DNA fragment coding  
for said protein, a method for preparing said protein or the  
salt thereof, and use of said protein and said DNA.

BACKGROUND OF THE INVENTION

15 PACAP was first isolated from the hypothalami of sheep  
as a peptide promoting adenylate cyclase activity of the  
pituitary glands [Biochemical Biophysical Research  
Communications, 164, 567-574 (1989)]. PACAP first isolated  
20 was one consisting of 38 amino acid residues. However, the  
presence of PACAP consisting of 27 residues on the N-  
terminal side was also revealed. Both are nearly equal in  
adenylate cyclase activating ability to each other. The  
former is referred to as PACAP38, and the latter is  
25 referred to as PACAP27. The expression of a PACAP having  
the same structure as that of sheep was also proved in  
humans, which suggested that PACAPs are important peptides  
conserved beyond species [Biochem. Biophys. Res. Commun.,  
166, 81-89 (1990)]. For the distribution thereof in  
30 organs, they are observed not only in the brain  
hypothalami, but also in the pituitary glands, the testes

and the adrenals (Endocrinology, 129, 2787-2789). At present, PACAPs such as PACAP27 to PACAP38 (U.S. Patent No. 5,128,242) and PACAP23 to PACAP26 (European Patent Publication No. 0467279A3) have been reported.

5       Physiological actions of PACAPs diversely varies according to their occurrence sites. Various actions of the PACAPs as described below have hitherto been reported:

(1) Promotion of cAMP production in primary culture cells of the rat pituitary glands [A. Miyata et al.,  
10 Biochem. Biophys. Res. Commun., 164, 567-574 (1989)];

(2) Promotion of secretion of GH, ACTH, PRL and LH in the rat pituitary gland superfusion process [A. Miyata et al., Biochem. Biophys. Res. Commun., 164, 567-574 (1989)];

(3) Production of cAMP in adrenomedullary  
15 chromaffinoma-derived cells PC12h and promotion of neurite outgrowth [T. Watanabe et al., Biochem. Biophys. Res. Commun., 173, 252-258 (1990), and K. Okazaki et al., FEBS Letters, 298, 49-56 (1992)];

(4) Promotion of interleukin-6 production in pituitary  
20 gland culture cells [I. Tatsuno et al., Endocrinology, 129, 1797-1804 (1991)]; and

(5) Promotion of cAMP production in primary culture of rat astrocytes and promotion of action preventing nerve cell death [Biochem. Biophys. Res. Commun., 168, 1027-1033  
25 (1990)].

In order for PACAP to exhibit its action, the presence

of a receptor specific for PACAP in target organs and cells is indispensable.

Receptor binding experiments using radioactive iodine-labeled PACAP27 ( $[^{125}\text{I}]$  PACAP27) have proved the presence of  
5 a PACAP receptor. Namely, when a membrane fraction prepared from a tissue is mixed with  $[^{125}\text{I}]$  PACAP27 and reacted for an appropriate period of time, binding of  $[^{125}\text{I}]$  PACAP27 to the membrane fraction is observed. This binding is inhibited by unlabeled PACAP27 or PACAP38, but not  
10 inhibited by VIP, an analogous peptide of the PACAPs. This result suggests that a substance specifically binding to the PACAPs occurs in the tissue. Such binding activity is highest in membrane fractions of the brain hypothalami, and also observed in the pituitary glands, the adrenals and the  
15 like [Endocrinology, 127, 272-277 (1990)]. Further, a body of PACAP binding activity observed in membrana cerebri fractions, namely a receptor, is deduced to be a protein having a molecular weight of 57,000 from a technique (so-called affinity-label experiment) comprising binding  $[^{125}\text{I}]$   
20 PACAP27 to the membrana cerebri fraction, crosslinking  $[^{125}\text{I}]$  PACAP27 and the body of its binding activity with a crosslinking reagent, then subjecting the product to polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate, and analyzing by autoradiography  
25 [Biochem. Biophys. Res. Commun., 171, 838-844 (1990)].

It is expected that clarification of some fundamental

properties of this specific receptor allows elucidation of additional various properties of the PACAP receptor to proceed more than before. In particular, cloning of cDNA coding for the receptor protein and structure analysis

5 thereof enable elucidation of the mechanism of its mutual interaction with a ligand, production of receptor-agonists and antagonists and detailed analysis of sites of action by in situ hybridization using said cDNA. Although cloning of the VIP, secretin and growth hormone releasing factor  
10 receptor proteins cDNA has been reported, the cloning of the PACAP receptor has not. These three kinds of bioactive peptides have also showed a capital similarity in the structures of their receptor proteins. For the PACAP receptors, however, cloning of cDNA has hitherto not been  
15 carried out.

Recently, the following five documents reported amino acid sequences for a rat PACAP receptor protein and nucleotide sequences of DNAs coding for the protein

[Document 1: Biochemical and Biophysical Research  
20 Communications, 194, 1, pp.133-143, 1993; Document 2: Federation of European Biochemical Societies (FEBS), 329, 1 and 2, pp. 99-105; Document 3: Proceedings of the National Academy of Science, USA, 90, pp. 6345-6349, 1993; Document 4: Nature, 365, pp. 170-175, 1993 and Document 5: Neuron,  
25 11, pp.333-342, 1993). Among them, the amino acid sequences and the nucleotide sequences described in the

Documents 1, 2, 4 and 5 are identified with the amino acid sequence for a rat PACAP receptor protein and with the nucleotide sequence for a DNA coding for the protein. The amino acid sequence described in Document 3 is different  
5 from the amino acid sequence of the present invention for a rat PACAP receptor protein in one amino acid, and the nucleotide sequence of Document 3 is also different from the nucleotide sequence of the present invention in one nucleotide. All of the five documents were published after  
10 June 24, 1993 which is one of the priority dates of the present invention.

In general, when specific binding substances such as receptors are purified, affinity column chromatography applying its mutual interaction with the specific binding  
15 substance (for example, ligands for receptors) are frequently used. A process using an affinity column in which a ligand is fixed on a carrier is simplest. However, many successful examples of complicated affinity chromatography are known in which the specific mutual  
20 interaction between avidin and biotin is utilized for purification of receptors. This process comprises synthesizing a biotinylated ligand in which biotin is bound to an appropriate site, and specifically capturing a receptor on a carrier on which avidin is fixed through the  
25 biotinylated ligand [Methods in Enzymology, 184, 244-274 (1990)]. This process suffers from the problem of

designing the biotinylated ligand having affinity for both the receptor and avidin, and examination is required in purifying PACAP receptor.

PACAP38 and PACAP27 are peptides represented by the following amino acid sequences, respectively:

PACAP38

His Ser Asp Gly Ile Phe Thr Asp Ser Tyr Ser Arg Tyr  
1 5 10  
Arg Lys Gln Met Ala Val Lys Lys Tyr Leu Ala Ala Val  
10 15 20 25  
Leu Gly Lys Arg Tyr Lys Gln Arg Val Lys Asn Lys-NH<sub>2</sub>  
30 35  
(SEQ ID NO: 46-NH<sub>2</sub>)

PACAP27

His Ser Asp Gly Ile Phe Thr Asp Ser Tyr Ser Arg Tyr  
15 1 5 10  
Arg Lys Gln Met Ala Val Lys Lys Tyr Leu Ala Ala Val  
15 20 25  
Leu-NH<sub>2</sub>  
20 (SEQ ID NO: 47-NH<sub>2</sub>)

SUMMARY OF THE INVENTION

In order to further elucidate the properties of the PACAP receptor protein which is a giant protein molecule and to collect useful information for development of drugs, purification of said protein, structure analysis by cDNA  
25 cloning and construction of its expression system are

indispensable. As described above, the presence of a protein showing high affinity for the PACAP, namely the PACAP receptor protein, has been known in the animal tissues. However, no report has so far been made that the  
5 PACAP receptor protein has yet to be obtained.

An object of the present invention is to purify the PACAP receptor protein and to clone a DNA coding for the PACAP receptor protein. If detailed information about the facts of the structure and functions of said protein is  
10 obtained, not only development of diagnostic methods for neuropathy such as Alzheimer's disease induced by a decrease in PACAP concentration is enabled by detecting the PACAP concentration in vivo, but also compounds activating PACAP receptor other than the known PACAP proteins or  
15 compounds antagonizing binding of a PACAP to a PACAP receptor can be enabled by using the PACAP receptor protein and the DNA coding for said protein. In addition, gene therapeutic composition for neuropathy such as Alzheimer's disease can be enabled by using said DNA.

20 The present inventors conducted intensive investigations, in view of the above-mentioned situation. As a result, bovine PACAP receptor protein was prepared unexpectedly efficiently by affinity chromatography using biotinylated PACAPs (particularly, biotinylated PACAP27).  
25 Further, synthetic DNA was prepared as a probe, based on the N-terminal amino acid sequence of the purified bovine

PACAP receptor protein, and a bovine brain cDNA library was screened to clone cDNA of bovine PACAP receptor. As a result, the present inventors first succeeded in cloning a bovine cDNA encoding the receptor protein for PACAP from the bovine brain cDNA library and in determining a nucleotide sequence of a translation region thereof.

Further, the present inventors elucidated the amino acid sequence of bovine PACAP receptor protein from this cDNA, and succeeded in pioneering the mass production thereof by recombinant technology.

Furthermore, the present inventors based on the similarity of the structure of PACAPs to that of VIP, secretin and growth hormone releasing factor, and deduced that receptors for the PACAPs would also show a similar structure to these, from the fact that the receptors already elucidated extremely resemble in structure among VIP, secretin and growth hormone releasing factor. Then, using as a probe cDNA of the VIP receptor having a higher similarity in structure as a ligand, cDNA of PACAP receptors was screened by homology screening. As a result, the present inventors first succeeded in cloning cDNA coding for rat PACAP receptor protein from a rat brain cDNA library, and in determining a nucleotide sequence of a translation region thereof. Further, the present inventors elucidated the amino acid sequence of rat PACAP receptor protein from this cDNA, and succeeded in pioneering the



mass production thereof by recombinant technology.

In addition, the present inventors succeeded in cloning cDNA coding for human PACAP receptor protein from a human pituitary cDNA library, using as a probe synthetic  
5 DNA prepared based on the amino acid sequence (sequence consisting of 16 amino acids) on the N-terminal side of the purified bovine PACAP receptor protein, and in determining a nucleotide sequence of a translation region thereof. Then, the present inventors elucidated the amino acid  
10 sequence of human PACAP receptor protein from this cDNA, produced this in large amounts by recombinant technology, and succeeded in pioneering the screening of compounds activating PACAP receptors or compounds antagonizing PACAP receptors by use of human PACAP receptor protein thus  
15 produced.

Namely, the present invention provides:

- (1) A receptor protein capable of binding a PACAP or a salt thereof;
- (2) The receptor protein of (1), wherein the receptor  
20 is endogenous to rat, bovine or human;
- (3) The receptor protein of (1) which comprises an amino acid sequence containing at least one member selected from the group consisting of the amino acid sequences of  
25 SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11 and SEQ ID NO: 12 or a

salt thereof;

(4) The receptor protein of (1) which comprises an amino acid sequence containing the amino acid sequence of SEQ ID NO: 13 or a salt thereof;

5 (5) The receptor protein of (1) which comprises an amino acid sequence having 90 to 100% homology as determined by sequence analysis with at least one member selected from the group consisting of the amino acid sequences of SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18,  
10 SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26 and SEQ ID NO: 28 or a salt thereof;

(6) The receptor protein of (1) which comprises an amino acid sequence selected from the group consisting of the amino acid sequences of SEQ ID NO: 14, SEQ ID NO: 15,  
15 SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28 and SEQ ID NO: 29 or a salt thereof;

(7) A receptor fragment containing a sufficient  
20 portion of the receptor of (1) to bind PACAP or a salt thereof;

(8) The receptor fragment of (7) selected from the group consisting of

(i) peptides having the amino acid sequence consisting  
25 of the 38th to 164th, 223rd to 232nd, 303rd to 317th or 416th to 424th amino acid residues of SEQ ID NO: 15,

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T0330: T45500

(ii) peptides having the amino acid sequence consisting of the 38th to 164th, 223rd to 232nd, 303rd to 317th or 388th to 397th amino acid residues of SEQ ID NO: 17,

5 (iii) peptides having the amino acid sequence consisting of the 20th to 146th, 205th to 214th, 286th to 299th or 369th to 378th amino acid residues of SEQ ID NO: 19,

(iv) peptides having the amino acid sequence  
10 consisting of the 20th to 146th, 205th to 214th, 286th to 299th or 397th to 406th amino acid residues of SEQ ID NO: 21, and

(v) peptides having the amino acid sequence consisting of the 78th to 204th, 263rd to 272nd, 342nd to 357th or  
15 427th to 436th amino acid residues of SEQ ID NO: 23; or a salt thereof;

(9) An isolated DNA coding for a receptor protein capable of binding a PACAP;

(10) The DNA of (9) wherein the receptor protein--  
20 comprises the amino acid sequence of SEQ ID NO: 14, SEQ ID NO 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO 18, SEQ ID NO: 19, SEQ ID NO 20, SEQ ID NO: 21, SEQ ID NO 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO 25, SEQ ID NO: 26, SEQ ID NO 27, SEQ ID NO: 28 or SEQ ID NO 29;

25 (11) The DNA of (9) comprising the nucleotide sequence of SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO 32,

(13) A vector containing the DNA of (9);

(14) A transformant containing the DNA of (9);

(15) A method for preparing the receptor protein or the salt thereof of (1) comprising cultivating a  
5 transformant containing a DNA encoding said protein under conditions suitable for expression of said protein and recovering said protein;

(16) A method for purifying the receptor protein or the salt thereof of (1) comprising subjecting a sample  
10 containing unpurified receptor protein to affinity chromatography using a biotinylated PACAP;

(17) The method of (16) comprising the steps of:

(a) preparing a membrane protein fraction from an animal tissue or cell,

15 (b) solubilizing the membrane protein fraction obtained in step (a),

(c) subjecting the solubilized membrane protein fraction obtained in step (b) to anion exchange chromatography and/or hydroxyapatite chromatography, and

20 (d) subjecting the active fraction obtained in step (c) to affinity chromatography using a biotinylated PACAP;

(18) The method of (17), in which the animal tissue is a bovine cerebrum;

(19) A method for preparing the receptor protein or  
25 the salt thereof of (1) comprising condensing a partial peptide fragment or a single amino acid corresponding to a

portion of the protein as claimed in claim 1 with a residual moiety, and removing a protective group as so desired when the product has the protective group, until said protein is obtained;

5       (20) A diagnostic composition for neuropathy comprising the PACAP receptor protein or the salt thereof of (1), or the receptor fragment or the salt thereof of (7);

10       (21) The diagnostic composition of (20) which is a diagnostic composition for Alzheimer's disease;

      (22) A gene therapeutic composition comprising the DNA of (9);

15       (23) The gene therapeutic composition of (22) to be administered to a patient whose an amount of PACAP receptor protein is decreased, to increase the amount of PACAP receptor protein;

20       (24) A method of diagnosis for neuropathy comprising contacting a sample to be tested with a receptor protein capable of binding a PACAP protein and measuring the amount of PACAP binding to the receptor protein;

      (25) The method of diagnosis of (24), wherein the receptor protein is a receptor fragment of (7);

25       (26) The method of (24) wherein a decrease in PACAP concentration is an indication of the presence of Alzheimer's disease;

      (27) A method of using the DNA of (9) to transform a

cell;

(28) The method of (27) wherein the cell is transformed in vitro;

(29) The method of (27) wherein the cell is  
5 transformed in vivo;

(30) The method of (27), in which the expression of the DNA increases the amount of PACAP receptor protein;

(31) A method for determining

(i) an effect of a test compound on PACAP receptor activity  
10 comprising comparing PACAP receptor activities in cases of (a) and (b);

(a) contacting PACAP receptor with a PACAP;

(b) contacting PACAP receptor with a PACAP and a test compound, or

15 (ii) an effect of a test compound on binding of PACAP to PACAP receptor comprising comparing an amount of binding of PACAP to PACAP receptor in cases of (a) and (b);

(a) contacting PACAP receptor with a PACAP;

(b) contacting PACAP receptor with a PACAP and a test  
20 compound;

(32) The method of (31) wherein the PACAP receptor is a protein of (1);

(33) The method of (31) wherein the PACAP receptor is a receptor fragment of (7);

25 (34) The method of (31) wherein the PACAP receptor is a protein produced by cultivating a transformant containing

the DNA of (9);

(35) The method of (31) which is a method for screening a compound activating PACAP receptor or a compound antagonizing binding of a PACAP to a PACAP  
5 receptor;

(36) An assay for quantifying a test compound's effect

(i) on PACAP receptor activity comprising comparing an amount of PACAP receptor activation in cases of (a) and  
10 (b);

(a) contacting PACAP receptor with a PACAP;

(b) contacting PACAP receptor with a PACAP and a test compound, or

(ii) on binding of PACAP to PACAP receptor comprising  
15 comparing an amount of binding of PACAP to PACAP receptor in cases of (a) and (b);

(a) contacting PACAP receptor with a PACAP;

(b) contacting PACAP receptor with a PACAP and a test compound;

20 (37) A compound or a salt thereof obtained by the method of (31);

(38) The compound or a salt thereof of (37) which is a compound activating PACAP receptor or a compound antagonizing binding of a PACAP to a PACAP receptor;

25 (39) A pharmaceutical composition for neuropathy comprising an effective amount of the compound or the salt

thereof of (37);

(40) The pharmaceutical composition of (39), wherein the neuropathy is Alzheimer's disease;

(41) An antibody to a receptor protein capable of  
5 binding a PACAP, a partial peptide thereof or a salt thereof;

(42) The antibody of (41) which is a monoclonal antibody selected from the group consisting of PRN1-25a, PRN1-109a and PRN1-159a;

10 (43) Hybridoma which produces a monoclonal antibody of (42);

(44) A signal peptide selected from the group of peptides consisting of a peptide which has 1st to 37th amino acid sequence of SEQ ID NO:15, a peptide which has  
15 1st to 37th amino acid sequence of SEQ ID NO:17, a peptide which has 1st to 19th amino acid sequence of SEQ ID NO:19, a peptide which has 1st to 19th amino acid sequence of SEQ ID NO:21, a peptide which has 1st to 77th amino acid sequence of SEQ ID NO:23, a peptide which has 1st to 77th  
20 amino acid sequence of SEQ ID NO:25, a peptide which has 1st to 77th amino acid sequence of SEQ ID NO:27, a peptide which has 1st to 77th amino acid sequence of SEQ ID NO:29, a peptide which has 58th to 77th amino acid sequence of SEQ ID NO:23, a peptide which has 58th to 77th amino acid  
25 sequence of SEQ ID NO:25, a peptide which has 58th to 77th amino acid sequence of SEQ ID NO:27 and a peptide which has



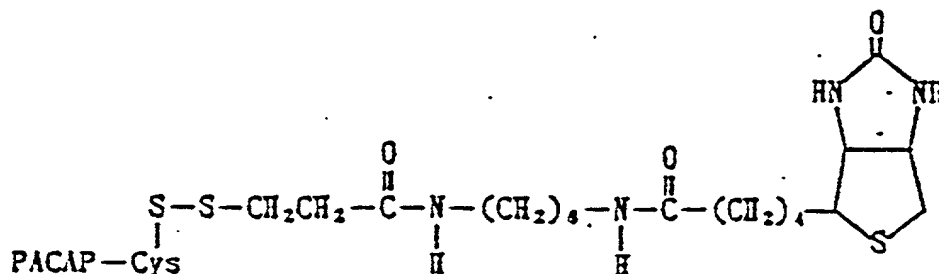
58th to 77th amino acid sequence of SEQ ID NO:29; or a salt thereof;

(45) A DNA which codes for a peptide of (44);

(46) A DNA of (45) which is selected from the group  
5 consisting of a DNA which has 1st to 111th nucleotide  
sequence of SEQ ID NO:30, a DNA which has 1st to 111th  
nucleotide sequence of SEQ ID NO:31, a DNA which has 1st to  
57th nucleotide sequence of SEQ ID NO:32, a DNA which has  
1st to 57th nucleotide sequence of SEQ ID NO:33, a DNA  
10 which has 1st to 231st nucleotide sequence of SEQ ID NO:34,  
a DNA which has 1st to 231st nucleotide sequence of SEQ ID  
NO:35, a DNA which has 1st to 231st nucleotide sequence of  
SEQ ID NO:36, a DNA which has 1st to 231st nucleotide  
sequence of SEQ ID NO:37, a DNA which has 172nd to 231st  
15 nucleotide sequence of SEQ ID NO:34, a DNA which has 172nd  
to 231st nucleotide sequence of SEQ ID NO:35, a DNA which  
has 172nd to 231st nucleotide sequence of SEQ ID NO:36 and  
a DNA which has 172nd to 231st nucleotide sequence of SEQ  
ID NO:37;

20 (47) A biotinylated PACAP;

(48) The biotinylated PACAP of (47) which is  
represented by the following formula:



(49) The biotinylated PACAP of (47) or (48), in which the PACAP is PACAP27; and

(50) A method for preparing the biotinylated PACAP of (47) comprising reacting a PACAP derivative in which a cysteine residue is introduced into the carboxyl terminus of a PACAP with a biotinylating reagent.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a restriction enzyme cleavage map of a bovine PACAP receptor cDNA clone, wherein A indicates *Ava*II, Ac indicates *Acc*I, B indicates *Bam*HI, Ba indicates *Bal*I, and S indicates *Sma*I;

Fig. 2 shows a nucleotide sequence of a bovine cDNA clone, pBPR-T, encoding receptor protein for PACAP and the predicted amino acid sequence. A signal sequence is deduced to be cleaved at that position indicated by the upward arrow  $\blacktriangle$ . Disappearance of the region between the open triangles  $\triangle$  is observed for pBPR-TD;

Fig. 3 shows a nucleotide sequence of a bovine cDNA clone, pBPR-T, encoding receptor protein for PACAP and the predicted amino acid sequence. A signal sequence is deduced to be cleaved at the position indicated by the upward arrow  $\blacktriangle$ . Insertion was observed at the position indicated by the open triangle  $\triangle$  for pBPR-TD;

Fig. 4 shows the N-terminal amino acid sequence of bovine PACAP receptor protein and an amino acid sequence

5 deduced from pBPR-T and pBPR-TD for comparison;

Fig. 5 shows graphs in which the degree of hydrophobicity of bovine PACAP receptor protein, encoded by pBPR-T and pBPR-TD, is shown as an index. A and B corresponds to pBPR-T and pBPR-TD, respectively. The numerals 1 to 7 indicate transmembrane domains presumed from the degree of hydrophobicity. The upward arrow ▲ indicates the position of a sequence which does not exist in pBPR-TD, but exists in pBPR-T;

15 Fig. 6 shows a restriction enzyme cleavage map of a rat PACAP receptor cDNA clone, wherein N indicates NcoI, P indicates PstI, and B indicates BamHI;

Fig. 7 shows a nucleotide sequence of rat PACAP receptor cDNA contained in pRPACAPR46-5 and an amino acid sequence of a translation frame derived therefrom. A signal sequence is deduced to be cleaved at the position indicated by the upward arrow ▲. Insertion is observed at the position indicated by the open triangle △ for pRPACAPR12;

Fig. 8 is a continuation of Fig. 7;

25 Fig. 9 shows a nucleotide sequence of rat PACAP receptor cDNA contained in pRPACAPR12 and an amino acid sequence of a translation frame derived therefrom. A signal sequence is deduced to be cleaved at the position indicated by the upward arrow ▲. The sequence between the open triangles △ is a sequence not existing in pRPACAPR46-5 and

30

characteristic of pRPACAPR12;

Fig. 10 is a continuation of Fig. 9;

Fig. 11 shows the N-terminal amino acid sequence of bovine PACAP receptor protein and the N-terminal amino acid  
5 sequence of rat PACAP receptor protein for comparison;

Fig. 12 shows a restriction enzyme cleavage map of a human PACAP receptor cDNA clone, wherein N indicates NcoI, ScI indicates SacI, Bg indicates BglII, Hp indicates HpaI, ScII indicates SacII, ET22 indicates EcoT22I, and Bs  
10 indicates BspEI;

Fig. 13 shows a nucleotide sequence of human PACAP receptor Type I-A cDNA coded with pTS847-1 and an amino acid sequence of a translation frame derived therefrom;

Fig. 14 shows the N-terminal amino acid sequence of  
15 bovine PACAP receptor protein and the N-terminal amino acid sequence deduced from human PACAP receptor protein cDNA for comparison;

Fig. 15 shows nucleotide sequences of pHPR15A, pHPR55A and pHPR66P encoding a portion of human PACAP receptor Type  
20 I-B, Type I-B2 and Type I-C respectively and predicted amino acid sequences of a translation frame. The region between two arrows shows an insertion sequence into human PACAP receptor Type I-A;

Fig. 16 shows a nucleotide sequence of cDNA of human  
25 PACAP receptor Type I-B and a predicted amino acid sequence of a translation frame. An underlined region is a sequence

inserted by an alternative splicing;

Fig. 17 shows a nucleotide sequence of cDNA of human PACAP receptor Type I-B2 and a predicted amino acid sequence of a translation frame. An underlined region is a sequence inserted by an alternative splicing;

Fig. 18 shows a nucleotide sequence of cDNA of human PACAP receptor Type I-C and a predicted amino acid sequence of a translation frame. An underlined region is a sequence inserted by an alternative splicing;

Fig. 19 shows graphs in which the degree of hydrophobicity of rat PACAP receptor protein encoded by pRPACAPR46-5 and pRPACAPR12 is shown as an index. A and B correspond to pRPACAPR46-5 and pRPACAPR12, respectively. The numerals 1 to 7 indicate portions deduced to be domains passing through a cell membrane from the degree of hydrophobicity. The upward arrow ▲ indicates the position of a sequence which does not exist in pRPACAPR46-5, but exists in pRPACAPR12;

Fig. 20 shows an amino acid sequence of rat PACAP receptor protein encoded by pRPACAPR46-5, and an amino acid sequence of rat VIP receptor protein for comparison. A group of amino acids 1 to 5 shown in the upper portion of the figure are regarded as equivalent to one another. Residues in which agreement is observed, including these amino acids, are given asterisks (\*). The upper lines indicate the amino acid sequence of the PACAP receptor

protein encoded by pRPACAPR46-5, and the lower lines indicate the sequence of rat VIP receptor. The numerals given above and under the respective sequences indicate the positions from the N-termini;

5        Fig. 21 shows a graph in which the degree of hydrophobicity of human PACAP receptor protein coded with pTS847-1 is shown as an index. The numerals 1 to 7 indicate portions deduced to be domains passing through a cell membrane from the degree of hydrophobicity;

10       Fig. 22 shows amino acid sequences of human PACAP receptor protein, bovine PACAP receptor protein and rat PACAP receptor protein for comparison. The arrow indicates a cleavage site of a signal peptide;

15       Fig. 23 is a series of graphs in which the degree of hydrophobicity of bovine PACAP receptor protein encoded by pBPR-T is shown as an index;

      Fig. 24 is a series of graphs in which the degree of hydrophobicity of bovine PACAP receptor protein encoded by pBPR-TD is shown as an index;

20       Fig. 25 is a series of graphs in which the degree of hydrophobicity of rat PACAP receptor protein encoded by pRPACAPR46-5 is shown as an index;

      Fig. 26 is a series of graphs in which the degree of hydrophobicity of rat PACAP receptor protein encoded by  
25 pRPACAPR12 is shown as an index;

      Fig. 27 is a series of graphs in which the degree of

hydrophobicity of human PACAP receptor protein encoded by pTS847-1 is shown as an index;

Fig. 28 is a graph showing absorption curves of biotinated PACAP27 by HPLC. Peak 1 of (A) indicates a peak of PACAP27-Cys, peak 3 of (B) indicates a peak of biotinated PACAP27-Cys, and peak 2 of (C) indicates a peak of the biotinating reagent;

Fig. 29 is a graph showing results of the antagonistic binding experiments of PACAP27 (O) and biotinated PACAP27 (●). The numerals on the abscissa indicate the concentrations (log M) of PACAP27 and biotinated PACAP27, and the numerals on the ordinate indicate the binding (%) of [ $^{125}$ I]-PACAP27, taking the specific binding as 100, when each peptide is added so as to give the final concentrations on the abscissa;

Fig. 30 is a graph showing Scatchard plot analysis of results of the saturation binding experiments of purified bovine PACAP receptor protein and membrane binding bovine receptor protein using [ $^{125}$ I]-PACAP27. Kd indicates the dissociation constant;

Fig. 31 is a graph showing results of the antagonistic binding experiment of purified bovine PACAP receptor protein. The numerals on the abscissa indicate the concentrations (log M) of PACAP38, PACAP27 and VIP, and the numerals on the ordinate indicate the binding (%) of [ $^{125}$ I]-PACAP27, taking the specific binding as 100, when each

peptide is added so as to give the final concentrations on the abscissa;

Fig. 32 shows analysis results of purified bovine PACAP receptor protein by polyacrylamide electrophoresis in the presence of sodium dodecylsulfate;

Fig. 33 is a graph showing results of the saturation binding experiment in a membrane fraction of CHO cells transfected with the bovine PACAP receptor protein cDNA(pBPR-T). The numerals on the abscissa indicate the concentration of [ $^{125}$ I]-PACAP27 added, and the numerals on the ordinate indicate the concentration of [ $^{125}$ I]-PACAP27 specifically bound to the membrane fraction;

Fig. 34 is a graph showing Scatchard plot in the membrane fraction of CHO cells transfected with the bovine PACAP receptor protein cDNA (pBPR-T);

Fig. 35 is a graph showing results of the competitive binding experiments of PACAP27, PACAP38 and VIP to [ $^{125}$ I]-PACAP27 in the membrane fraction of CHO cells transfected with the bovine PACAP receptor protein cDNA (pBPR-T). The numerals on the abscissa indicate the concentrations (log M) of PACAP27, PACAP38 and VIP, and the numerals on the ordinate indicate the binding (%) of [ $^{125}$ I]-PACAP27, taking the specific binding as 100, when each peptide is added so as to give the final concentrations on the abscissa;

Fig. 36 is a graph showing changes in the amounts of intracellular cyclic AMP of CHO cells transfected with the



bovine PACAP receptor protein cDNA (pBPR-T) produced by PACAP27, PACAP38 and VIP. The numerals on the abscissa indicate the concentrations (log M) of PACAP27, PACAP38 and VIP, and the numerals on the ordinate indicate the relative concentrations of cyclic AMP in the transformant CHO cells treated with peptides having respective concentrations, taking the concentration of cyclic AMP in untreated transformant CHO cells as 1;

Fig. 37 is a graph showing changes in the amounts of intracellular inositol phosphate of CHO cells transfected with the bovine PACAP receptor protein cDNA (pBPR-T) produced by PACAP27, PACAP38 and VIP. The numerals on the abscissa indicate the concentrations (log M) of PACAP27, PACAP38 and VIP, and the numerals on the ordinate indicate the relative concentrations of inositol phosphate in transformant CHO cells treated with peptides having respective concentrations, taking the concentration of inositol phosphate in untreated transformant CHO cells as 1;

Fig. 38 shows results of northern hybridization using RNA prepared from the rat brains, lungs, livers, kidneys and testes, and a rat PACAP receptor protein cDNA probe. The bands represent that the RNA prepared from the rat brains, lungs, livers, kidneys and testes, and the rat PACAP receptor protein cDNA probe exhibit cross reaction. The numerals on the left indicate the size of a molecular

weight marker;

Fig. 39 shows results of measurements of the radioactivity, wherein each column indicates the binding of each CHO cell product with [ $^{125}$ I]-PACAP27 when cultured in each of the following combinations:

Column 1: untreated CHO cells + [ $^{125}$ I]-PACAP27

Column 2: untreated CHO cells + [ $^{125}$ I]-PACAP27 + cold PACAP27

Column 3: untreated CHO cells + [ $^{125}$ I]-PACAP27 + cold VIP

10 Column 4: pRPR3-A-introduced CHO cells + [ $^{125}$ I]-PACAP27

Column 5: pRPR3-A-introduced CHO cells + [ $^{125}$ I]-PACAP27 + cold PACAP27

Column 6: pRPR3-A-introduced CHO cells + [ $^{125}$ I]-PACAP27 + cold VIP

15 Column 7: pRPR4-B-introduced CHO cells + [ $^{125}$ I]-PACAP27

Column 8: pRPR4-B-introduced CHO cells + [ $^{125}$ I]-PACAP27 + cold PACAP27

Column 9: pRPR4-B-introduced CHO cells + [ $^{125}$ I]-PACAP27 + cold VIP

20 Column 10: rat VIP receptor cDNA-introduced CHO cells + [ $^{125}$ I]-PACAP27

Column 11: rat VIP receptor cDNA-introduced CHO cells + [ $^{125}$ I]-PACAP27 + cold PACAP27

25 Column 12: rat VIP receptor cDNA-introduced CHO cells + [ $^{125}$ I]-PACAP27 + cold VIP;

Fig. 40 are graphs showing results of competitive

binding experiments. (A) is a graph showing results of the competitive binding experiments of PACAP27 and VIP to [ $^{125}$ I]-PACAP27 in a membrane fraction of CHO cells transfected with rat PACAP receptor protein cDNA (pRPR3-A).

5 (B) is a graph showing results of the competitive binding experiments of PACAP27 and VIP to [ $^{125}$ I]-PACAP27 in a membrane fraction of CHO cells transfected with rat PACAP receptor protein cDNA (pRPR4-B). The numerals on the abscissa indicate the concentrations (log M) of PACAP27 and  
10 VIP, and the numerals on the ordinate indicate the binding (%) of [ $^{125}$ I]-PACAP27, taking the specific binding as 100, when each peptide is added so as to give the final concentrations on the abscissa;

Fig. 41 shows the binding of [ $^{125}$ I]-PACAP27 in the  
15 membrane fraction of CHO cells transfected with the rat PACAP receptor protein cDNA (pRPR3-A). A indicates CHO cells transfected with a rat PACAP receptor protein cDNA (pRPR3-A), and B indicates CHO cells transfected with a rat PACAP receptor protein cDNA (pRPR4-B). The numerals on the  
20 abscissa indicate sample Nos. of transformant CHO cells obtained by separating single clone-derived colonies, and the numerals on the ordinate indicate the binding (cpm) of [ $^{125}$ I]-PACAP27;

Fig. 42 shows results of examination of  
25 reproducibility of clones having much [ $^{125}$ I] binding in Fig. 37. The numerals 1 and 2 on the abscissa indicate

untreated CHO cells, the numerals 3 and 4 VIP cDNA-introduced CHO cells, the numerals 5 and 6 clone B1, the numerals 7 and 8 clone B2, the numerals 9 and 10 clone B17, the numerals 11 and 12 clone A6, the numerals 13 and 14 clone A12, and the numerals 15 and 16 clone A15. The numerals on the ordinate indicate the binding (cpm) of [<sup>125</sup>I]-PACAP27;

Fig. 43 are graphs showing the changes in the amounts of intracellular cyclic AMP. The upper graph (type I-A) indicates changes in the amounts of intracellular cyclic AMP of CHO cells transfected with the rat PACAP receptor protein cDNA (pBPR-T) produced by PACAP27, PACAP38 and VIP. The numerals on the abscissa indicate the concentrations (log M) of PACAP27, PACAP38 and VIP, and the numerals on the ordinate indicate the concentration (ratio (%) to the maximum production amount) of intracellular cyclic AMP.

The lower graph (type I-B) indicates changes in the amounts of intracellular cyclic AMP of CHO cells transfected with the rat PACAP receptor protein cDNA (pBPR-TD) produced by PACAP27, PACAP38 and VIP. The numerals on the abscissa indicate the concentrations (log M) of PACAP27, PACAP38 and VIP, and the numerals on the ordinate indicate the concentration (ratio (%) to the maximum production amount) of intracellular cyclic AMP;

Fig. 44 shows the amount of rat PACAP receptor protein expressed in transformant Sf9 cells with baculoviruses.

Sf9 was infected with 10 clones of recombinant viruses at the stage of primary plaque measurement, and 4 days after culture, the binding of the cells to [ $^{125}$ I]-PACAP27 was assayed. The numerals on the abscissa indicate sample Nos. 5 of the transformant Sf9 cells. Sample Nos. 1 to 3 indicate transformant Sf9 cells containing rat PACAP receptor protein cDNA introduced by a vector modified from pRPR3-A, sample Nos. 4 to 6 indicate transformant Sf9 cells containing rat PACAP receptor protein cDNA introduced by a 10 vector modified from pRPR4-B, and sample No. 7 indicates uninfected Sf9 cells (control). Cold (-) on the ordinate indicates the binding of each sample to [ $^{125}$ I]-PACAP27 when only 100 pM [ $^{125}$ I]-PACAP27 is added, and cold (+) indicates the binding of each sample to [ $^{125}$ I]-PACAP27 when 100 pM 15 [ $^{125}$ I]-PACAP27 and 1  $\mu$ M unlabeled PACAP27 are added;

Fig. 45 shows the amount of human PACAP receptor protein expressed in transformant Sf9 cells with baculoviruses. Sf9 was infected with 10 clones of recombinant viruses at the stage of primary purification, 20 and cultured for 4 days after infection. The binding of [ $^{125}$ I]-PACAP27 on the cells was assayed. The numerals on the abscissa indicate sample Nos., and the numerals on the ordinate indicate the amount of [ $^{125}$ I]-PACAP27 binding. Sample No. 0 indicates uninfected Sf9 cells (control);

25 Fig. 46 is a graph showing Scatchard plot in a membrane fraction of CHO-K1 cells transfected with pTS849

which expresses human PACAP receptor protein.

Fig. 47 is a graph showing results of the competitive binding experiments of PACAP27 ( $\square$ ), PACAP38 (O) and VIP ( $\Delta$ ) to [ $^{125}$ I]-PACAP27 in a membrane fraction of CHO cells

5 transfected with pTS849 which expresses human PACAP receptor protein. The numerals on the abscissa indicate the concentrations (log M) of PACAP27, PACAP38 and VIP, and the numerals on the ordinate indicate the binding (%) of [ $^{125}$ I]-PACAP27, taking the specific binding as 100, when  
10 each peptide is added so as to give the final concentrations on the abscissa;

Fig. 48 is a graph showing changes in the amounts of intracellular cyclic AMP of CHO cells transfected with human PACAP receptor protein cDNA (pTS847-1) produced by  
15 PACAP27 ( $\square$ ), PACAP38 (O) and VIP ( $\Delta$ ). The numerals on the abscissa indicate the concentrations (log M) of PACAP27, PACAP38 and VIP, and the numerals on the ordinate indicate the relative concentrations of cyclic AMP in transformant CHO cells treated with peptides having respective  
20 concentrations, taking the concentration of inositol phosphate in untreated transformant CHO cells as 1;

Fig. 49 shows results of Northern hybridization using RNA prepared from the human brain, lung, liver, thymus, spleen, pancreas and placenta, and a human PACAP receptor  
25 protein cDNA probe. The bands represent that the RNA prepared from the human brain, lung, liver, thymus, spleen,

pancreas placenta, and the human PACAP receptor protein  
cDNA probe exhibit cross reaction. The numerals on the  
left indicate the size of a molecular weight marker;

Fig. 50 shows results of norther hybridization using  
RNA prepared from the rat olfactory bulbs, amygdalae,  
cerebral basal ganglia, hippocampi, hypothalami, cerebral  
cortices, medulla oblongatas, cerebellums, vertebrae and  
pituitary glands, and a rat PACAP receptor protein cDNA  
probe. The bands represent that the RNA prepared from the  
rat olfactory bulbs, amygdalae, cerebral basal ganglia,  
hippocampi, hypothalami, cerebral cortices, medulla  
oblongatas, cerebellums, vertebrae and pituitary glands,  
and the rat PACAP receptor protein cDNA probe exhibit cross  
reaction. The numerals on the left indicate the size of a  
molecular weight marker.

Fig. 51-1 and Fig. 51-2 show a formula of the compound  
which was found by the screening using the membrane fraction  
of Sf9 cells which expressed human PACAP receptor Type I-A  
by baculovirus.

Fig. 52 is a graph which shows a typical sample of  
screening of hybridomas.

Fig. 53 shows a detection of PACAP receptor by Western  
blotting with the antibody of the present invention. Lane  
1 shows rainbow coloured protein molecular weight markers  
and lane 2 shows a result of 320 ng of a membrane protein  
solubilized in an insect cell containing 20 ng of human

PACAP receptor.

Fig. 54 is a graph which shows that the antibody of the present invention inhibits binding of PACAP27 and PACAP receptor.

## 5 DETAILED DESCRIPTION OF THE INVENTION

The present inventors obtained cDNA clones of the PACAP receptor proteins from the bovine brain cDNA library. Of these,  $\lambda$ BPR35,  $\lambda$ BPR114 and  $\lambda$ BPR68 were cloned, and subcloned into pUC118 to obtain pBPR35, pBPR114 and pBPR68  
10 (Fig. 1). Further, pBPR35 and pBPR68 were recombined at the BamHI site to prepare pBPR-T having a complete translation frame. The complete primary structure of bovine PACAP receptor protein based on pBPR-T was deduced (Fig. 2, pBPR-T). 84 nucleotides were not present in  
15 pBPR114, compared with pBPR-T. This is considered to occur by alternative splicing in a transcription product from a common gene.

The PACAP receptor protein which does not contain 84 nucleotides can be prepared by recombining pBPR-T with  
20 pBPR114 at the BamHI and Ava II sites. The amino acid sequence of the recombinant protein was deduced (Fig. 3, pBPR-TD). The total number of amino acid residues and the molecular weight derived from these sequences are 513 residues (58.5 kilodaltons) for pBPR-T, and 485 residues  
25 (55.3 kilodaltons) for pBPR-TD. As to both the molecules, the N-terminal sequence up to the 37th Ala residue was



deduced to be a signal sequence for passing through a membrane.

Further, the N-terminal sequence after this processing completely agreed with the N-terminal amino acid sequence of bovine PACAP receptor protein purified in this invention (Fig. 4). Analysis of hydrophobicity based on the amino acid sequence revealed in the present invention proved that bovine PACAP receptor protein has 7 hydrophobic amino acid clusters considered to be transmembrane domains in tandem (Fig. 5). Such structural features are common to peptide receptor proteins of the G protein coupling type [European Journal of Pharmacology, 227, 1-8 (1992)].

The present inventors further cloned  $\lambda$ RPACAPR18,  $\lambda$ RPACAPR46,  $\lambda$ RPACAPR5 and  $\lambda$ RPACAPR12 as cDNA clones of the PACAP protein from the rat brain cDNA library, and subcloned into pCDNA I or pUC118 to obtain pRPACAPR18, pRPACAPR46, pRPACAPR5 and pRPACAPR12 (Fig. 6). Further, pRPACAPR46 and pRPACAPR5 were recombined at the BamHI site to prepare pRPACAPR46-5 having a complete translation frame. Based on these, two kinds of complete primary structures of rat PACAP receptor protein were deduced (Figs. 7 and 8, and Figs. 9 and 10). The first methionine in each sequence is considered to be an initiation codon. It is conceivable that the difference between two kinds of sequences shown in Figs. 7 and 8 and Figs. 9 and 10 arises by alternative splicing in a transcription product from a

common gene. The total number of amino acid residues and the molecular weight derived from these sequences are 467 residues (53.2 kilodaltons), and 495 residues (56.4 kilodaltons), respectively. As to both the molecules, the N-terminal sequence up to the 19th Ala residue was deduced to be a signal sequence for passing through a membrane. Further, the N-terminal sequence after this processing completely showed high homology with the N-terminal amino acid sequence of bovine PACAP receptor protein purified (Fig. 11).

In addition, the present inventors cloned  $\lambda$ #14 shown in Fig. 12 as a cDNA clone of the PACAP protein from the human pituitary cDNA library, and subcloned into pUC118 to obtain pTS847-1. Based on this, the complete primary structure of human PACAP receptor protein was deduced (Fig. 13). The first methionine in its sequence is considered to be an initiation codon. The total number of amino acid residues and the molecular weight derived from Fig. 13 are 525 residues and 59.3 kilodaltons, respectively. As to this molecule, the N-terminal sequence up to the 77th Ala residue was deduced to be a signal sequence for passing through a membrane. Further, the N-terminal sequence after this processing completely showed high homology with the N-terminal amino acid sequence of bovine PACAP receptor protein purified by Ohtaki et al (Fig. 14).

The present inventors found out that 84 nucleotides

were inserted at the same sites of both rat and bovine Type I-B of PACAP receptor, and therefore deduced that there would also be a similar insertion at the same site of a human PACAP receptor. The inventors prepared a primer from  
5 the sequence flanking to the deduced insertion site and conducted PCR methods. As a result, the present inventors succeeded in cloning a cDNA encoding the insertion region of subtype of a human PACAP receptor and in identifying the amino acid sequence of the insertion region, by applying  
10 PCR method to cDNAs of human pituitary and amygdaloid nucleus. The present inventors further succeeded in preparing a cDNA encoding whole length of subtype of a human PACAP receptor by recombining the above cDNAs with the cDNA of Type I-A of a human PACAP receptor. In more  
15 detail, the present inventors obtained pHPR15A, pHPR55A and pHPR66P as cDNA clones of the insertion region of a subtype of a human PACAP receptor by applying PCR method to cDNAs of human amygdaloid nucleus and human pituitary (Fig. 15). By recombining the clones with human PACAP receptor Type  
20 I-A at the recognition sites of HpaI and AvaII, a cDNA for each subtype was constructed. The nucleotide sequences of cDNAs of the constructed subtypes and the amino acid sequence deduced therefrom are shown in Figs. 16 to 18.

Analysis of hydrophobicity based on the amino acid  
25 sequence revealed in the present invention proved that rat PACAP receptor protein has 7 hydrophobic amino acid

clusters considered to be membrane permeable domains in tandem (Fig. 19(A) indicates a result of analysis of the protein shown in Figs. 7 and 8, and Fig. 19(B) indicates a result of analysis of the protein shown in Figs. 9 and 10).

5 Such structural features are common to peptide receptor proteins of the G protein binding type [European Journal of Pharmacology, 227, 1-8 (1992)]. The peptides or ligands were extremely similar in structure, and the result of comparison at the amino acid sequence level with the  
10 structure of a VIP receptor used as a cDNA probe for cloning revealed extremely high similarity. As a whole, the similarity of the N-terminal portions is very low, whereas regions containing the first to seventh membrane permeable domains and the C-terminal intracellular domains  
15 conversely showed high similarity. It was further revealed that human PACAP receptor protein also has 7 hydrophobic amino acid clusters considered to be membrane permeable domains in tandem (Fig. 21).

The amino acid sequences of bovine PACAP receptor  
20 protein, rat PACAP receptor protein and human PACAP receptor protein of the present invention showed very high homology (Fig. 22). All of these proteins were proved to have amino acid sequences represented by SEQ ID NOs: 1 to 12.

25 The present inventors named rat PACAP receptor protein having the amino acid sequence of SEQ ID NO: 19 "Type I-A",

and rat PACAP receptor protein having the amino acid sequence of SEQ ID NO: 21 "Type I-B". Bovine PACAP receptor protein having the amino acid sequence of SEQ ID NO: 15 is bovine PACAP receptor protein Type I-A

5 corresponding to rat PACAP receptor protein Type I-A of SEQ ID NO: 19, and bovine PACAP receptor protein having the amino acid sequence of SEQ ID NO: 17 is bovine PACAP receptor protein Type I-B corresponding to rat PACAP receptor protein Type I-B of SEQ ID NO: 21.

10 Human PACAP receptor protein having the amino acid sequence of SEQ ID NO: 23 is human PACAP receptor protein Type I-A corresponding to rat PACAP receptor protein Type I-A of SEQ ID NO: 19, and human PACAP receptor protein having the amino acid sequence of SEQ ID NO: 25 which clone  
15 is obtained by recombining pHPR15A is human PACAP receptor protein Type I-B. pHPR55A lacks 3 nucleotides, CAG, from pHPR15A, which lacks Ser as an amino acid. The human PACAP receptor protein having an amino acid sequence of SEQ ID NO:27 was named "Type I-B2" since the protein is  
20 thought to be a clone resulting from a sliding of the position of a splicing of Type I-B. Further, a human PACAP receptor protein having an amino acid sequence of SEQ ID NO:29, a recombinant clone of pHPR66P, which is thought to result from a transcription product of a common gene by an  
25 alternative splicing and the subtype was named Type I-C.

The origin of amino acid sequences of PACAP receptor

proteins and nucleotide sequences of DNAs coding for said proteins represented by SEQ ID NO used in this specification are as follows:

[SEQ ID NO: 1-SEQ ID NO: 12]

- 5        These indicate amino acid sequences which bovine, rat and human PACAP receptor proteins have in common;

[SEQ ID NO: 13]

         This indicates an N-terminal amino acid sequence of the purified bovine PACAP receptor protein;

- 10      [SEQ ID NO: 14]

         This indicates an amino acid sequence of a protein in which a signal peptide is deleted from bovine PACAP receptor protein Type I-A encoded by in pBPR-T;

[SEQ ID NO: 15]

- 15        This indicates an amino acid sequence of bovine PACAP receptor protein Type I-A encoded by in pBPR-T;

[SEQ ID NO: 16]

         This indicates an amino acid sequence of a protein in which a signal peptide is deleted from bovine PACAP receptor protein Type I-B encoded by in pBPR-TD;

- 20      [SEQ ID NO: 17]

[SEQ ID NO: 17]

         This indicates an amino acid sequence of bovine PACAP receptor protein Type I-B encoded by in pBPR-TD;

[SEQ ID NO: 18]

- 25        This indicates an amino acid sequence of a protein in which a signal peptide is deleted from rat PACAP receptor

protein Type I-A;

[SEQ ID NO: 19]

This indicates an amino acid sequence of rat PACAP  
receptor protein Type I-A;

5 [SEQ ID NO: 20]

This indicates an amino acid sequence of a protein in  
which a signal peptide is deleted from rat PACAP receptor  
protein Type I-B;

[SEQ ID NO: 21]

10 This indicates an amino acid sequence of rat PACAP  
receptor protein Type I-B;

[SEQ ID NO: 22]

This indicates an amino acid sequence of a protein in  
which a signal peptide is deleted from human PACAP receptor  
15 protein Type I-A;

[SEQ ID NO: 23]

This indicates an amino acid sequence of human PACAP  
receptor protein Type I-A;

[SEQ ID NO: 24]

20 This indicates an amino acid sequence of a protein in  
which a signal peptide is deleted from human PACAP receptor  
protein Type I-B;

[SEQ ID NO: 25]

This indicates an amino acid sequence of human PACAP  
25 receptor protein Type I-B;

[SEQ ID NO: 26]

This indicates an amino acid sequence of a protein in which a signal peptide is deleted from human PACAP receptor protein Type I-B2;

[SEQ ID NO: 27]

- 5        This indicates an amino acid sequence of human PACAP receptor protein Type I-B2;

[SEQ ID NO: 28]

- 10       This indicates an amino acid sequence of a protein in which a signal peptide is deleted from human PACAP receptor protein Type I-C;

[SEQ ID NO: 29]

This indicates an amino acid sequence of human PACAP receptor protein Type I-C;

[SEQ ID NO: 30]

- 15       This indicates a nucleotide sequence of cDNA coding for bovine PACAP receptor protein Type I-A;

[SEQ ID NO: 31]

This indicates a nucleotide sequence of cDNA coding for bovine PACAP receptor protein Type I-B;

- 20       [SEQ ID NO: 32]

This indicates a nucleotide sequence of cDNA coding for rat PACAP receptor protein Type I-A;

[SEQ ID NO: 33]

- 25       This indicates a nucleotide sequence of cDNA coding for rat PACAP receptor protein Type I-B;

[SEQ ID NO: 34]



This indicates a nucleotide sequence of cDNA coding  
for human PACAP receptor protein Type I-A;

[SEQ ID NO: 35]

This indicates a nucleotide sequence of cDNA coding  
5 for human PACAP receptor protein Type I-B;

[SEQ ID NO: 36]

This indicates a nucleotide sequence of cDNA coding  
for human PACAP receptor protein Type I-B2;

[SEQ ID NO: 37]

10 This indicates a nucleotide sequence of cDNA coding  
for human PACAP receptor protein Type I-C;

[SEQ ID NO: 38]

This indicates a nucleotide sequence of DNA (pBPR-T)  
containing a nucleotide sequence of cDNA coding for bovine  
15 PACAP receptor protein Type I-A;

[SEQ ID NO: 39]

This indicates a nucleotide sequence of DNA (pBPR-TD)  
containing a nucleotide sequence of cDNA coding for bovine  
PACAP receptor protein Type I-B;

20 [SEQ ID NO: 40]

This indicates a nucleotide sequence of DNA (pRPACAPR  
46-5) containing a nucleotide sequence of cDNA coding for  
rat PACAP receptor protein Type I-A;

[SEQ ID NO: 41]

25 This indicates a nucleotide sequence of DNA (pRPACAPR  
12) containing a nucleotide sequence of cDNA coding for rat

PACAP receptor protein Type I-B;

[SEQ ID NO: 42]

This indicates a nucleotide sequence of DNA (pTS847-1) containing a nucleotide sequence of cDNA coding for human

5 PACAP receptor protein Type I-A;

[SEQ ID NO: 43]

This indicates a nucleotide sequence of DNA containing a nucleotide sequence of cDNA coding for human PACAP receptor protein Type I-B;

10 [SEQ ID NO: 44]

This indicates a nucleotide sequence of DNA containing a nucleotide sequence of cDNA coding for human PACAP receptor protein Type I-B2;

[SEQ ID NO: 45]

15 This indicates a nucleotide sequence of DNA containing a nucleotide sequence of cDNA coding for human PACAP receptor protein Type I-C;

[SEQ ID NO: 46]

This indicates an amino acid sequence of PACAP38.

20 [SEQ ID NO: 47]

This indicates an amino acid sequence of PACAP27.

[SEQ ID NO: 48]

This indicates a nucleotide sequence of an oligonucleotide used for screening of cDNA coding for rat

25 PACAP receptor proteins Type I-A and Type I-B;

[SEQ ID NO: 49]

This indicates a nucleotide sequence of an oligonucleotide used for screening of cDNA coding for rat PACAP receptor proteins Type I-A and Type I-B.

[SEQ ID NO: 50]

5 This indicates an N-terminal amino acid sequence (sequence consisting of 16 amino acids) of bovine PACAP receptor protein.

[SEQ ID NO: 51]

10 This indicates a nucleotide sequence of an oligonucleotide used for screening of cDNA encoding bovine and human PACAP receptor proteins.

[SEQ ID NO: 52]

15 This indicates a nucleotide sequence of a primer prepared from cDNA encoding human PACAP receptor protein Type I-A.

[SEQ ID NO: 53]

This indicates a nucleotide sequence of a primer prepared from cDNA encoding human PACAP receptor protein Type I-A.

20 [SEQ ID NO: 54]

This indicates a nucleotide sequence of a probe prepared based on the nucleotide sequence at the insertion region of rat PACAP receptor protein Type I-B.

[SEQ ID NO: 55]

25 This indicates a nucleotide sequence of a probe prepared based on the nucleotide sequence at other

insertion region than rat PACAP receptor protein Type I-B.

When nucleotides, amino acids and so on are indicated by abbreviations in the specification and drawings, the abbreviations adopted by the IUPAC-IUB Commission on

5 Biochemical Nomenclature or commonly used in the art are employed. For example, the following abbreviations are used. When the amino acids are capable of existing as optical isomers, it is understood that the L-forms are represented unless otherwise specified.

- 10 DNA : Deoxyribonucleic acid  
cDNA : Complementary deoxyribonucleic acid  
A : Adenine  
T : Thymine  
G : Guanine  
15 C : Cytosine  
RNA : Ribonucleic acid  
mRNA : Messenger ribonucleic acid  
dATP : Deoxyadenosine triphosphate  
dTTP : Deoxythymidine triphosphate  
20 dGTP : Deoxyguanosine triphosphate  
dCTP : Deoxycytidine triphosphate  
ATP : Adenosine triphosphate  
EDTA : Ethylenediaminetetraacetic acid  
SDS : Sodium dodecyl sulfate  
25 EIA : Enzyme immunoassay  
Gly : Glycine

Ala : Alanine

Val : Valine

Leu : Leucine

Ile : Isoleucine

5 Ser : Serine

Thr : Threonine

Cys : Cysteine

Met : Methionine

Glu : Glutamic acid

10 Asp : Aspartic acid

Lys : Lysine

Arg : Arginine

His : Histidine

Phe : Phenylalanine

15 Tyr : Tyrosine

Trp : Tryptophan

Pro : Proline

Asn : Asparagine

Gln : Glutamine

20 Further, meanings of other abbreviations used in this  
specification are as follows:

VIP : Vasoactive intestinal peptide

Tris : Tris(hydroxymethyl)aminomethane

EDTA : Ethylenediaminetetraacetic acid

25 PMSF : Phenylmethylsulfonyl fluoride

BSA : Bovine serum albumin

CHAPS: 3-[(3-Cholamidopropyl)dimethylammonio]-1-  
propanesulfonate

Biotin-HSDP:

N-[6-(biotinamido)hexyl]-3'-(2'-pyridylthio)-  
propionic acid amide

5

"TM" used in this specification represents a  
registered trade mark.

The PACAP receptor proteins which are capable of  
binding a PACAP of the present invention may be derived  
10 from tissues of warm-blooded animals (for example, the  
cerebrums, pituitary glands and adrenals of rats, mice,  
hamsters, chickens, dogs, cats, sheep, monkeys, pigs,  
cattle or humans) or cells [for example, adrenal chromaffin  
cells, glial cells and established cell lines (such as PC12  
15 cells, NB-OK cells and AR4-2J cells)], or may be produced  
by chemical synthesis. Any proteins may be used as long as  
they have PACAP receptor activity ("PACAP receptor  
activity" means the action of specifically binding to the  
PACAPs). Examples thereof include proteins having amino  
20 acid sequences containing at least one member selected from  
the group consisting of the amino acid sequences  
represented by SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3,  
SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ  
ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11 and  
25 SEQ ID NO: 12. Such proteins include, for example,  
proteins having only the amino acid sequences of SEQ ID NO:

1 to SEQ ID NO: 12, respectively, and proteins in which amino acids and (or) peptides are further bound to said proteins at their N-terminal sites and (or) C-terminal sites. Preferable examples of such proteins include

5 proteins having amino acid sequences containing the amino acid sequences represented by SEQ ID NO:1 to SEQ ID NO:12. Specifically, they include proteins having the amino acid sequences represented by SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ  
10 ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28 or SEQ ID NO: 29. Preferably, the proteins having the amino acid sequences represented by SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16 or SEQ ID NO: 17 are  
15 bovine-derived proteins, the proteins having the amino acid sequences represented by SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20 or SEQ ID NO: 21 are rat-derived proteins, and the proteins having the amino acid sequences represented by SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25,  
20 SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28 or SEQ ID NO: 29 are human derived proteins.

The amino acid sequences represented by SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID  
25 NO: 10, SEQ ID NO: 11 and SEQ ID NO: 12 are amino acid sequences which the bovine-, rat- and human-derived PACAP

receptors which lack signal peptide, that is PACAP  
receptors having the amino acid sequences represented by  
SEQ ID NO: 14, 16, 18, 20, 22, 24, 26 or 28, have in common  
(Fig. 22). Further, as apparent from Fig. 22, the amino  
5 acid sequences of the PACAP receptor proteins exhibit high  
homology among species of warm-blooded animals, so that  
proteins having usually 90-100%, preferably 95-100%, and  
more preferably 97-100% homology with the amino acid  
sequence(s) represented by SEQ ID NO: 14, SEQ ID NO: 16,  
10 SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24,  
SEQ ID NO: 26 and/or SEQ ID NO: 28 are also included in the  
PACAP receptor proteins of the present invention.

Further, for example, proteins having amino acid  
sequences containing the amino acid sequence represented by  
15 SEQ ID NO: 13 can also be used. Examples of such proteins  
include proteins having only the amino acid sequence of SEQ  
ID NO: 13, and proteins in which amino acids or peptides  
are further bound to said proteins at their C-terminal  
sites. Specifically, proteins having the amino acid  
20 sequence of SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16 or  
SEQ ID NO: 17 are used. In particular, bovine-derived  
proteins are preferred.

Furthermore, the PACAP receptor proteins of the  
present invention also comprise proteins in which the N-  
25 terminal Met residues are protected with protective groups  
(for example, C<sub>1-6</sub> acyl groups such as formyl and acetyl),



proteins in which peptide bond between the 9th Lys residues and the 10th Glu residues in the amino acid sequences represented by SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26 or SEQ ID NO: 28 are cleaved in vivo and the Glu residues are pyroglutaminated, proteins in which side chains of amino acids in molecules are protected with appropriate protective groups, and conjugated proteins such as so-called glycoproteins to which sugar chains are bound.

10 As used herein, "PACAP receptor protein" also includes a salt of said protein. Salts of the PACAP receptor proteins used in the present invention include, for example, salts with inorganic acids (such as hydrochloric acid, phosphoric acid, hydrobromic acid and sulfuric acid) 15 and salts with organic acids (such as acetic acid, formic acid, propionic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid and benzenesulfonic acid).

20 In producing the proteins of the present invention, when the proteins are extracted from animal tissues or cells, methods for purifying proteins can generally be employed. In particular, the proteins of the present invention are of the membrane binding type, so that 25 solubilization of membrane fractions is required. Concrete purifying methods are shown below:

(1) Preparation of a membrane fraction suspension

A membrane fraction suspension can be prepared by treating an animal tissue, for example, by the method described in Biochem. Biophys. Res. Commun., 172, 709-714  
5 (1990) or a method based thereon.

(2) Solubilization of a desired protein fraction from the membrane fraction suspension

The membrane fraction obtained in (1) described above is solubilized by the method described in Biochem. Biophys.  
10 Res. Commun., 172, 709-714 (1990) or a method based thereon. Examples of solubilizing reagents which can be used therein include detergents having skeletons of bile acid (such as digitonin and CHAPS) and nonionic detergents (such as TWEEN 20<sup>TM</sup> and TRITON-X<sup>TM</sup>). Specifically, the  
15 membrane fraction suspension is diluted with an appropriate buffer (for example, Tris buffer) to give a protein concentration of 0.1 to 5.0 mg/ml, preferably 0.5 to 2.0 mg/ml, and more preferably 1.0 mg/ml, the above-mentioned solubilizing reagent is added thereto to yield a  
20 concentration of 0.1 to 5.0%, preferably 0.5 to 2.0%, and more preferably 1.0%, and the mixture is stirred usually for 10 minutes to 72 hours, preferably for 30 minutes to 24 hours, followed by ultracentrifugation to obtain a supernatant thereof. The presence or absence of a desired  
25 protein can be detected by measuring the activity of said protein. For example, PACAP receptor activity can be

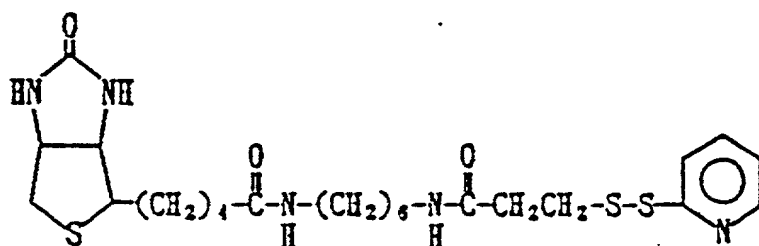
supernatant thereof. The presence or absence of a desired protein can be detected by measuring the activity of said protein. For example, PACAP receptor activity can be measured by the method described in Biochem. Biophys. Res.

5 Commun., 172, 709-714 (1990) or methods based thereon.

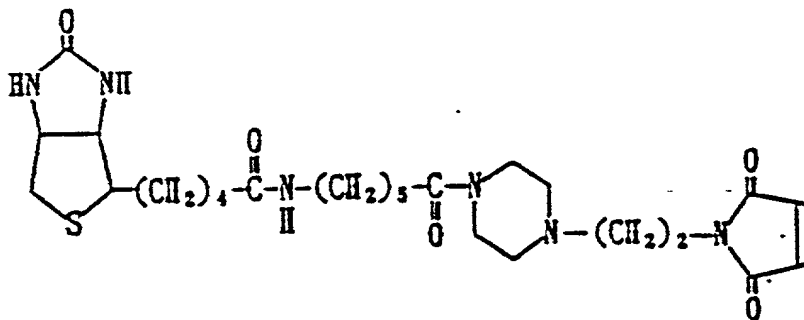
(3) Purification of the desired protein from the solubilized sample

Purification of the desired protein from the solubilized sample obtained in (2) described above can be  
10 conducted by anion-exchange chromatography [for example, DEAE-TOYOPEARL™ (Tosoh)], hydroxyapatite chromatography [for example, HCA-100™ (Mitsui Toatsu Chemicals)], affinity chromatography [for example, avidin-agarose (Pierce)], gel filtration [for example, SUPERROSE™  
15 (Pharmacia)], etc. under appropriate conditions. In particular, the methods for producing the desired protein in the present invention are characterized in that the desired protein can be purified at high efficiency by use of affinity chromatography using the "biotinylated PACAPs"  
20 first discovered as ligands in the present invention. As said PACAPs, PACAP27 to PACAP38 described in EP-A-0404652 and PACAP23 to PACAP26 described in EP-A-0467279 are used. In particular, PACAP27 and PACAP38 are preferred. Examples of methods for biotinylating the PACAP include the method  
25 of introducing a cysteine residue into the carboxyl terminus of the PACAP to synthesize a PACAP derivative, and

easily binding a commercially available biotinylating reagent through the cysteine residue. As the derivatives, for example, PACAP27-Cys and PACAP38-Cys are used. The derivatives can be produced by methods known in the art or methods based thereon, for example, solid phase methods. The biotinylating reagents used include, for example, the following reagents:



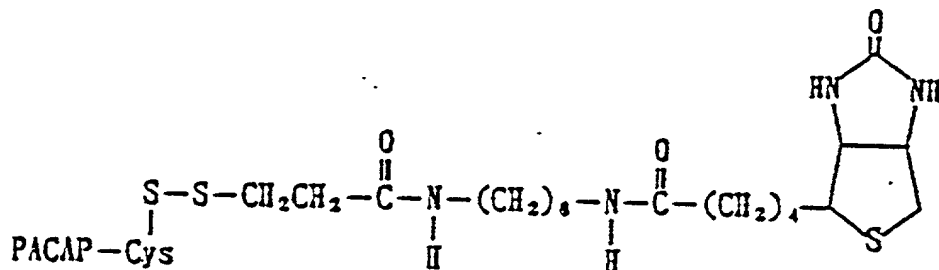
N-[6-(Biotinamido)hexyl]-3'-(2'-pyridylthio)-propionic acid amide (Cat. No. 21341, Pias)



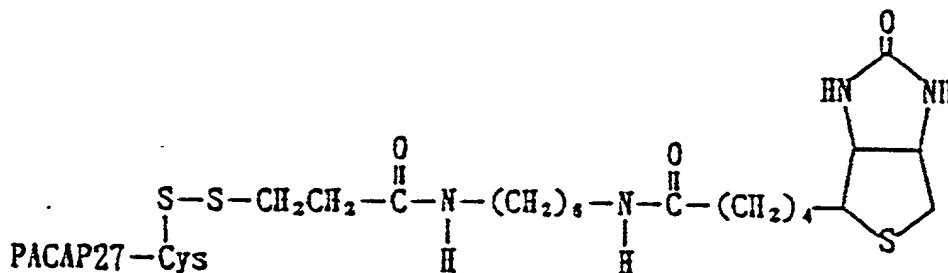
6-[N' {2-(N-Maleimido)ethyl}-N-piperazinylamido]hexyl biotinamide (Code No. 344-06391, Dojin Kagaku Kenkyusho)

The binding of the derivatives to the biotinylating reagents can be carried out by the method described in Biochem. Biophys. Res. Commun., 184, 123-160 (1990) or methods based thereon.

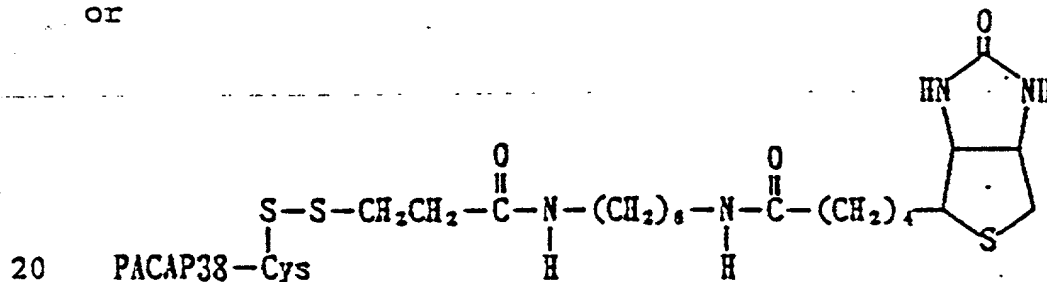
Examples of the biotinylated PACAPs of the present invention include ones represented by the following formula:



10 In particular, one represented by



or



are preferred.

Of these biotinylated PACAPs, the method for producing biotinylated PACAP27 is described in detail in Example 1 (5) set out below. Other ligands can also be produced in accordance with the method of Example 1 (5-1).

The biotinylated PACAPs of the present invention thus obtained have binding ability to both PACAP receptor proteins and avidin. They can be therefore used for many purposes such as staining and flow cytophotometry of cells and tissues, as well as purification of PACAP receptor proteins.

On the other hand, when the proteins of the present invention are produced by chemical synthesis, they are produced by methods known in the art or methods based thereon. For example, either solid phase synthesis methods or liquid phase synthesis methods may be employed. Namely, the desired peptides can be produced by condensing partial peptides or amino acids which can constitute the proteins of the present invention with residual moieties, and eliminating protective groups when the products have the protective groups. Known condensing methods and elimination of the protective groups include, for example, methods described in (1) to (5) given below:

- (1) M. Bodansky and M. A. Ondetti, Peptide Synthesis, Interscience Publishers, New York (1966);
- (2) Schroeder and Luebke, The Peptide, Academic Press, New York (1965);
- (3) N. Izumiya et al., Peptide Gosei no Kiso to Jikken (Fundamentals and Experiments of Peptide Synthesis), Maruzen (1985);
- (4) H. Yazima, S. Sakakibara et al., Seikagaku Jikken

5 Koza (Course of Biochemical Experiments), 1, Chemistry of  
Proteins IV, 205 (1977); and

(5) Zoku Iyakuhiin no Kaihatu (Development of Drugs)  
second series), 14, Peptide Synthesis, supervised by H.  
Yazima, Hirokawa Shoten.

10 After reaction, the PACAP receptor proteins of the  
present invention can be isolated by combinations of usual  
purification methods such as solvent extraction,  
distillation, reprecipitation, column chromatography, liquid  
chromatography, and recrystallization.

15 When the PACAP receptor proteins obtained by the above-  
mentioned methods are free forms, they can be converted to  
appropriate salts by known methods. Conversely, when the  
proteins are obtained in the salt state, they can be  
converted to the free forms by known methods.

20 The receptor fragments capable of binding a PACAP of  
the present invention may be any peptides e.g., a receptor  
fragment or a truncated receptor, as long as they have  
PACAP receptor activity. For example, sites of PACAP  
receptor protein molecules exposed out of cell membranes  
are used. Specifically, they are partial peptides deduced  
25 to be extracellular regions in hydrophobic plot analysis  
(Fig. 23 to Fig. 27). Examples thereof include:

(1) peptides having the amino acid sequence consisting  
of the 38th to 164th, 223rd to 232nd, 303rd to 317th or  
416th to 424th amino acid residues of SEQ ID NO: 15 (bovine

30

PACAP receptor protein Type I-A) (Fig. 23);

(2) peptides having the amino acid sequence consisting of the 38th to 164th, 223rd to 232nd, 303rd to 317th or 388th to 397th amino acid residues of SEQ ID NO: 17 (bovine  
5 PACAP receptor protein Type I-B) (Fig. 24);

(3) peptides having the amino acid sequence consisting of the 20th to 146th, 205th to 214th, 286th to 299th or 369th to 378th amino acid residues of SEQ ID NO: 19 (rat  
PACAP receptor protein Type I-A) (Fig. 25);

10 (4) peptides having the amino acid sequence consisting of the 20th to 146th, 205th to 214th, 286th to 299th or 397th to 406th amino acid residues of SEQ ID NO: 21 (rat  
PACAP receptor protein Type I-B) (Fig. 26); and

(5) peptides having the amino acid sequence consisting  
15 of the 78th to 204th, 263rd to 272nd, 342nd to 357th or 427th to 436th amino acid residues of SEQ ID NO: 23 (human  
PACAP receptor protein Type I-A) (Fig. 27).

The receptor fragments capable of binding a PACAP can be produced by known methods for synthesizing the peptides  
20 of (1) to (5) described above or by cleaving the PACAP receptor proteins with appropriate peptidases.

Salts of the receptor fragments capable of binding a PACAP used in the present invention include, for example, salts with inorganic acids (such as hydrochloric acid,  
25 phosphoric acid, hydrobromic acid and sulfuric acid) and salts with organic acids (such as acetic acid, formic acid,



propionic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid and benzenesulfonic acid).

- 5       The DNAs coding for the PACAP receptor proteins of the present invention may be any, as long as they have nucleotide sequences coding for the PACAP receptor proteins. Namely, the DNAs encoding the PACAP receptor proteins of the present invention may be any of cDNA,
- 10   genomic DNA and synthetic DNA. Further, the DNAs may be ones encoding the PACAP receptor proteins derived from any warm-blooded animals (for example, rats, mice, hamsters, chickens, dogs, cats, sheep, monkeys, pigs, cattle, humans and so on), namely the above-mentioned PACAP receptor
- 15   proteins of the present invention. Specifically, the DNAs having the nucleotide sequences of SEQ ID NO: 30 to SEQ ID NO: 45, respectively, are used. Screening of the DNAs can be conducted by general genetic engineering techniques or methods based thereon, for example, based on the examples 2
- 20   to 4 given below.

Expression vectors for the PACAP receptor proteins can be produced by (a) restricting desired DNA fragments from the DNAs encoding the PACAP receptor proteins, and (b) ligating the DNA fragments downstream from promoters in

25   appropriate vectors.

The cloned DNAs encoding the PACAP receptor proteins

can be used as such, or after digestion with restriction enzymes or addition of linkers if desired, depending on their purpose.

The DNA may have ATG as a translation initiation codon on the 5'-terminal side, and TAA, TGA or TAG as a translation termination codon on the 3'-terminal side. The translation initiation codon and translation termination codon may be added by use of an appropriate synthetic DNA adaptor. A promoter is further ligated upstream therefrom to express the DNA.

The vectors include plasmids derived from Escherichia coli (for example, pBR322, pBR325, pUC12 and pUC13), plasmids derived from Bacillus subtilis (for example, pUB110, pTP5 and pC194), plasmids derived from yeast (for example, pSH19 and pSH15, bacteriophages (for example,  $\lambda$  phage), and viruses such as retroviruses, vaccinia viruses and baculoviruses.

As the promoter used in the present invention, any promoter is available as long as it is suitable for expression corresponding to a host cell used for the gene expression.

When the host cell used for transformation is Escherichia, a trp promoter, a lac promoter, a recA promoter, a  $\lambda P_L$  promoter or an lpp promoter is preferred. When the host cell is Bacillus, an SP01 promoter, an SP02 promoter or a penP promoter is preferred. When the host

cell is yeast, a PHO5 promoter, a PGK promoter, a GAP promoter or an ADH promoter is preferred.

When the host cell is an animal cell, a SV40-derived promoter, a CMV-derived promoter, a retrovirus promoter, a  
5 metallothionein promoter, etc. are each usable.

An enhancer is also effectively utilized for expression.

Using the vectors containing the DNAs coding for the PACAP receptor proteins thus constructed, transformants are  
10 prepared.

Examples of the host cells include Escherichia, Bacillus, yeast, insects and animal cells.

Examples of the above-mentioned Escherichia include E. coli K12·DH1 [Proc. Natl. Acad. Sci. U.S.A., 60, 160  
15 (1968)], JM103 [Nucleic Acids Research, 9, 309 (1981)], JA221 [Journal of Molecular Biology, 120, 517, (1978)], HB101 [Journal of Molecular Biology, 41, 459 (1969)] and C600 [Genetics, 39, 440 (1954)].

Examples of the above-mentioned Bacillus include  
20 Bacillus subtilis MI114 [Gene, 24, 255 (1983)] and 207-21 [Journal of Biochemistry, 95, 87 (1984)].

Examples of the above-mentioned yeast include Saccharomyces cerevisiae AH22, AH22R<sup>-</sup>, NA87-11A, DKD-5D and  
20B-12.

25 Examples of the insects include larvae of silk worms [Maeda et al., Nature, 315, 592 (1985)].

Examples of the animal cells include monkey cell COS-7, Vero, Chinese hamster cell (CHO), mouse L cell and human FL cell.

The transformation of the above-mentioned Escherichia  
5 is conducted, for example, according to the method described in Proc. Natl. Acad. Sci. U.S.A., 69, 2110 (1972), Gene, 17, 107 (1982) or the like.

The transformation of the Bacillus is carried out, for example, according to the method described in Molecular &  
10 General Genetics, 168, 111 (1979) or the like.

The transformation of the yeast is performed, for example, according to the method described in Proc. Natl. Acad. Sci. U.S.A., 75, 1929 (1978).

The transformation of the insects is conducted, for  
15 example, according to the method described in Bio/Technology, 6, 47-55 (1988) or the like.

The transformation of the animal cells is carried out, for example, according to the method described in Virology,  
52, 456 (1973).

20 Thus, the transformants transformed with the expression vectors containing the cDNAs coding for the PACAP receptor proteins are obtained.

When the bacterial transformants are cultivated, a liquid medium is typically used for cultivation. Carbon  
25 sources, nitrogen sources, inorganic compounds and other nutrients necessary for growth of the transformants are

contained therein. Examples of the carbon sources include glucose, dextrin, soluble starch and sucrose. Examples of the nitrogen sources include inorganic or organic materials such as ammonium salts, nitrates, corn steep liquor,

5 peptone, casein, meat extracts, soybean meal and potato extract solution. The inorganic compounds include, for example, calcium chloride, sodium dihydrogenphosphate and magnesium chloride. Yeast, vitamins and growth promoting factors, etc. may be further added.

10 The pH of the medium is preferably about 5 to about 8.

When the Escherichia transformants are cultivated, M9 medium containing glucose and Casamino Acids [Miller, Journal of Experiments in Molecular Genetics, 431-433, Cold Spring Harbor Laboratory, New York (1972)] is preferably  
15 used to cultivate the transformants. In order to allow the promoters to act more efficiently, for example, drugs such as 3 $\beta$ -indolyl acrylic acid may be added thereto if necessary.

The Escherichia transformants are usually cultivated  
20 at about 15 to 43°C for about 3 to 24 hours with aeration or agitation if necessary.

The Bacillus transformants are usually cultivated at about 30 to 40°C for about 6 to 24 hours with aeration or agitation if necessary.

25 When the yeast transformants are cultivated, a preferred medium is Burkholder minimum medium [K. L.

Bostian, Proc. Natl. Acad. Sci. U.S.A., 77, 4505 (1980)] or SD medium containing 0.5% Casamino Acids [G. A. Bitter et al., Proc. Natl. Acad. Sci. U.S.A., 81, 5330 (1984)]. The pH of the medium is preferably adjusted to about 5 to 8.

5 The cultivation is usually carried out at about 20 to 35°C for about 24 to 72 hours with aeration or agitation if necessary.

When the insect transformants are cultivated, examples of media used include Grace's insect medium [(T. C. C. Grace, Nature, 195, 788 (1962)] supplemented with an additive such as 10% inactivated bovine serum. The pH of the medium is preferably adjusted to about 6.2 to 6.4. The cultivation is usually carried out at about 27°C for about 3 to 5 days with aeration or agitation if necessary.

15 When the animal cell transformants are cultured, examples of media used include MEM medium containing about 5 to 20% fetal calf serum [Science, 122, 501 (1952)], DMEM medium [Virology, 8, 396 (1959)], RPMI 1640 medium [Journal of the American Medical Association, 199, 519 (1967)] and 20 199 medium [Proceeding of the Society for the Biological Medicine, 73, 1 (1950)]. The pH is preferably about 6 to 8. The cell culture is usually carried out at about 30 to 40°C for about 15 to 60 hours, with aeration or agitation if necessary.

25 The isolation and purification of the PACAP receptor proteins from the above-mentioned culture products can be

carried out, for example, according to the following method.

When the PACAP receptor protein is to be extracted from cultured cells, the cells are collected by methods known in the art after cultivation. Then, the collected cells are suspended in an appropriate buffer solution, and disrupted by ultrasonic treatment, lysozyme treatment and/or freeze-thawing thereby releasing the PACAP receptor protein, followed by centrifugation to obtain a crude extract of the PACAP receptor protein. The buffer solution may contain a protein denaturant such as urea or guanidine hydrochloride, or a detergent such as Triton X-100.

When the PACAP receptor protein is secreted in the culture solution, a supernatant is separated from the cells by methods known in the art after termination of cultivation, and then collected. The separation and purification of the PACAP receptor protein contained in the culture supernatant or the extract thus obtained can be carried out by appropriate combinations of well-known separating and purifying methods. These known separating and purifying methods include methods utilizing a difference in solubility such as salting-out and solvent precipitation, methods mainly utilizing a difference in molecular weight such as dialysis, ultrafiltration, gel filtration and SDS-polyacrylamide gel electrophoresis, methods utilizing a difference in electric charge such as

ion-exchange chromatography, methods utilizing specific  
affinity such as affinity chromatography, methods utilizing  
a difference in hydrophobicity such as reverse phase high  
performance liquid chromatography, and methods utilizing a  
5 difference in isoelectric point such as isoelectric point  
electrophoresis.

Before or after purification, an appropriate protein  
modifying enzyme can also be reacted with the PACAP  
receptor protein produced by a recombinant to arbitrarily  
10 modify the protein or to partially eliminate a polypeptide  
therefrom. The protein modifying enzymes used include  
trypsin, chymotrypsin, arginyl endopeptidase and protein  
kinase.

The activity of the PACAP receptor proteins thus  
15 obtained can be measured by enzyme immunoassays. When the  
products have dephosphorylation activity, the measurement  
can also be conducted based upon said activity.

In the PACAP receptor proteins of the present  
invention, the amino acid sequences thereof may be  
20 partially modified (addition, elimination or substitution  
with other amino acids).

The PACAP receptor proteins and the DNAs coding for  
said proteins of the present invention thus obtained can be  
used for (i) acquisition of antibodies and antisera, (ii)  
25 construction of expression systems of recombinant receptor  
proteins, (iii) development of receptor binding assay



preparation of probes and PCR primers in gene diagnosis,  
and (vi) detection of PACAPs or PACAP receptors in vivo.  
In particular, the information hitherto obtained suggests  
that the PACAPs are deeply related to the functions of the  
5 hypothalamus-pituitary gland system, the sympathetic nerve  
system and the central nerve system. Accordingly,  
elucidation of the structure and properties of the PACAP  
receptors can contribute to development of unique drugs  
acting on these systems.

10 The PACAP receptor proteins, the PACAP receptor  
fragments and the DNAs encoding said proteins of the  
present invention can be used as follows (1) to (3)

(1) The PACAPs are known to exhibit functions such as  
protection of nerve cells and growth maintenance of the  
15 nerve cells in vivo. A decrease in PACAP concentration in  
vivo is therefore considered to induce death of the nerve  
cells and to cause neuropathy such as Alzheimer's disease.

Accordingly, for the PACAP receptor proteins of the present  
invention which specifically react with the PACAPs, the  
20 partial peptides thereof or the salts thereof, the PACAP  
concentration in vivo can be determined high sensitively,  
so that they can be effectively used as diagnostic  
composition for neuropathy such as Alzheimer's disease.

When the PACAP receptor proteins of the present invention,  
25 the partial peptides thereof or the salts thereof are used  
as diagnostic composition which can determine the PACAP

composition for neuropathy such as Alzheimer's disease.  
The diagnosis can be conducted by determining an amount  
of PACAP which binds to PACAP receptor proteins, the  
partial peptides thereof or the salts thereof of the  
present invention when contacting the test sample with  
PACAP receptor proteins, the partial peptides thereof  
of the salts thereof of the present invention. When the  
PACAP receptor proteins of the present invention,  
the partial peptides thereof of the salts thereof are used  
as diagnostic composition which can determine the PACAP  
concentration in test samples, they can be used, for  
example, in combination with competitive assays. For  
example, the methods described in the following (i) or  
(ii), or methods based thereon can be used:

(i) Radioimmunoassay, edited by H. Irie, Kodansha  
(1974), and

(ii) Radioimmunoassay (second series), edited by H.  
Irie, Kodansha (1979)

Specifically, standard curves can be prepared by the  
receptor competitive binding experiment method described in  
Example 1 (3) given later, thereby measuring the PACAP  
concentration in test samples. The procedure of the method  
is shown below.

[Table 1]

5

A test sample is mixed  
with receptor protein  
solution [ $^{125}\text{I}$ ]PACAP27

or

A standard sample (un-  
labeled PACAP27) is  
mixed with receptor  
protein solution [ $^{125}\text{I}$ ]  
PACAP27



10

Incubated for 1 hour



15

Separation of binding ligands from free ligands:  
Filtration (a glass fiber filter treated with 0.3%  
polyethylene)



20

Measurement of the amount of binding ligands



25

The concentration of the test sample is determined from  
the standard curve obtained from the standard sample.

(2) In the case of a patient suffering from neuropathy (for example, Alzheimer's disease) in which the PACAP action is not sufficiently exhibited, because the PACAP can not be bound to the PACAP receptor in vivo due to a  
5 reduction in the amount of the PACAP receptor protein on the nerve cell membranes in vivo, causing the tendency of death of the nerve cells, the amount of the PACAP receptor protein in the nerve cells of the patient can be increased by (a) inserting the DNA of the present invention in the  
10 patient to express it, or by (b) inserting the DNA of the present invention in the nerve cells to express it, followed by implantation of the nerve cells in the patient, thereby sufficiently exhibiting the PACAP action. That is, the DNAs of the present invention can be used for gene  
15 therapy of neuropathy, because we can transform nerve cells in vitro or in vivo by using the DNAs of the present invention.

The above-mentioned gene therapy can be given according to methods known in the art. For example, they  
20 can be given orally as tablets, capsules, elixirs and microcapsules, or parenterally in the form of injections such as sterile solutions or suspensions with water or with pharmaceutically acceptable solutions other than water. For example, the DNAs of the present invention can be mixed  
25 with carriers, flavoring agents, excipients, vehicles, preservatives, stabilizing agents, binders, etc. in the

form of unit dosage required for generally admitted pharmaceutical practice to prepare preparations. The amount of active ingredients in these preparations is adjusted so as to obtain appropriate doses within specified

5 ranges. Additives which can be mixed with tablets, capsules, etc. include, for example, binders such as gelatin, corn starch, tragacanth and gum arabic; excipients such as crystalline cellulose; swelling agents such as corn starch, gelatin and alginic acid; lubricants such as

10 magnesium stearate; sweeteners such as sucrose, lactose and saccharine; and flavoring agents such as peppermint, acamono oil and cherry. When the preparation unit is in the capsule form, liquid carriers such as fat and oil may further be added to materials of the above-mentioned types.

15 Sterile compositions for injection can be formulated according to usual pharmaceutical practice such as dissolution or suspension of active substances and naturally occurring vegetable oils such as sesame oil and coconut oil in vehicles such as water for injection.

20 Aqueous solutions for injection include physiological saline and isotonic solutions containing glucose or other adjuvants (for example, D-sorbitol, D-mannitol and sodium chloride), and may be used in combination with appropriate solubilizing adjuvants such as alcohols (for example,

25 ethanol), polyalcohols (for example, polypropylene glycol and polyethylene glycol) and nonionic surface active agents

(for example, Polysolvate 80 and HCO-50). Oily solutions include sesame oil and soybean oil, and may be used in combination with solubilizing adjuvants such as benzyl benzoate, benzyl alcohol, etc. The preparations may  
5 further contain buffers (for example, phosphate buffer and sodium acetate buffer), soothing agents (for example, benzalkonium chloride and procaine hydrochloride), stabilizing agents (for example, human serum albumin and polyethylene glycol), preservatives (for example, benzyl  
10 alcohol and phenol), antioxidants, etc. The injections thus prepared are usually filled into appropriate ampuls. Although the dosage varies depending upon the symptom, the oral dosage is generally about 0.1 to 100 mg per day, preferably 1.0 to 50 mg, and more preferably 1.0 to 20 mg,  
15 for adults (taken as 60 kg). When the preparations are parenterally given, the dosage varies depending upon the object to which the preparations are given, the organ to which they are given, the symptom, the route of administration, etc. For example, when the preparations  
20 are given in the injection form, it is advantageous that they are intravenously injected in a dosage of about 0.01 to 30 mg per day, preferably 0.1 to 20 mg, and more preferably 0.1 to 10 mg, for adults (taken as 60 kg).

(3) Example 1 below and Fig. 31 have proved that the  
25 PACAP receptor proteins of the present invention bind to the PACAPs. Further, Examples 5, 7 and 8 have revealed

- that the DNAs coding for bovine, rat and human PACAP receptor proteins can be expressed on cell membranes, and the PACAP receptor proteins expressed can react with the PACAPs to increase the amount of cyclic AMP and (or) the concentration of inositol phosphate in cells. Further Example 11 has revealed that compounds inhibiting the binding of PACAPs to PACAP receptors can be screened by using the membrane fractions of the Sf9 cells in which the human PACAP receptor is expressed by use of Baculoviridae.
- 10 Accordingly, the present invention gives a method for determining
- (i) an effect of a test compound on PACAP receptor activity comprising comparing PACAP receptor activities in cases of (a) and (b);
- 15 (a) contacting PACAP receptor with a PACAP;
- (b) contacting PACAP receptor with a PACAP and a test compound, or
- (ii) an effect of a test compound on binding of PACAP to PACAP receptor comprising comparing an amount of binding of
- 20 PACAP to PACAP receptor in cases of (a) and (b);
- (a) contacting PACAP receptor with a PACAP;
  - (b) contacting PACAP receptor with a PACAP and a test compound.

- The present invention further gives an assay for
- 25 quantifying a test compound's effect
- (i) on PACAP receptor activity comprising comparing an

amount of PACAP receptor activation in cases of (a) and  
5 (b);

(a) contacting PACAP receptor with a PACAP;

(b) contacting PACAP receptor with a PACAP and a test  
compound, or

(ii) on binding of PACAP to PACAP receptor comprising  
10 comparing an amount of binding of PACAP to PACAP receptor  
in cases of (a) and (b);

(a) contacting PACAP receptor with a PACAP;

(b) contacting PACAP receptor with a PACAP and a test  
compound.

15 As the PACAP receptor in the above screening method,  
the PACAP receptor of the present invention, the receptor  
fragment of the present invention or the PACAP receptor  
produced by cultivating a transformant containing the DNA  
encoding the PACAP receptor of the present invention.

20 Compounds or their salts obtained by the above  
screening method include compounds activating PACAP receptor  
or compounds antagonizing binding of a PACAP to a PACAP  
receptor.

25 As the above mentioned, compounds activating PACAP  
receptors (for example, peptides, proteins and natural or  
nonnatural compounds), namely, PACAP receptor antagonists,  
or compounds antagonistically inhibiting the binding of  
PACAPs to receptors (for example, peptides, proteins and  
natural or nonnatural compounds), namely PACAP receptor



antagonists, can be screened by using the PACAP receptor  
5 proteins of the present invention, the partial peptides  
thereof or the salts thereof, or by the PACAP receptor  
proteins which are obtained by cultivating transformants  
containing a DNA encoding PACAP receptor protein. These  
10 PACAP receptor agonists or PACAP receptor antagonists can be  
further tested for use as drugs useful in protection of  
nerve cells and growth maintenance of nerve cells in vivo,  
for example, therapeutic composition for neuropathy such as  
Alzheimer's disease.

Until human PACAP receptor of the present invention  
15 was found, for example, when substances which inhibit a  
binding of PACAP to human PACAP receptor were screened, the  
following steps were necessary:

Obtaining a PACAP receptor of other than human such as  
20 bovine or porcine; screening substances which inhibit a  
binding of the bovine or porcine PACAP receptor and PACAP;  
and checking whether the picked substances have real  
affinity on human PACAP receptor.

Meanwhile, human PACAP receptor makes the screening of  
25 substances which inhibit binding of human PACAP receptor  
and PACAP easy and effective. The thus obtained PACAP  
receptor agonists or PACAP receptor antagonists may be  
further tested drugs useful for protecting nerve cells or  
maintaining growth of nerve cells in vivo such as for  
30 therapeutic composition for nervous diseases such as

Alzheimer's disease or for maintaining growth of nerve cell  
in vitro.

The screening methods of the present invention will be described below in detail.

5 (I) Methods for Screening Compounds Antagonistically  
Inhibiting the Binding of PACAPs to PACAP Receptors

PACAP receptor proteins used for screening are preferably membrane fractions of organs of warm-blooded animals. For example, human PACAP receptor protein  
10 expressed in large amounts by use of recombinants is suitable, because it is very difficult to obtain human-derived organs.

The above-mentioned methods are used for the production of the PACAP receptor proteins, and performed by  
15 expressing DNAs coding for said proteins in animal cells (for example, human cells) or insect cells. In particular, they are preferably expressed in the insect cells.

Complementary DNAs are used as the DNA fragments coding desired portions, but the DNA fragments are not  
20 necessarily limited thereto. For example, gene fragments or synthetic DNAs may be used. In order to introduce the DNA fragments coding for the PACAP receptor proteins into host cells and express them efficiently, it is preferred that said DNA fragments are ligated downstream from  
25 polyhedrin promoters of insect nuclear polyhedrosis viruses (NPVs) belonging to Baculoviridae. Vectors include two

viruses of *Autographa californica* NPV (AcNPV) belonging to Kinuwaba and *Bombyx mori* NPV (BmNPV) of silk worms.

Baculoviridae has cyclic double stranded DNA (130 kb), and shows no infectivity to spinal animals and plants at all.

5 Virus DNA is so long as 130 kb, so that it is difficult to directly insert the DNA fragment wanted to be expressed downstream from the polyhedrin promoter. Then, actually, a polyhedrin gene portion containing a promoter portion is cut out from a virus, and incorporated in an E. coli vector  
10 such as pUC18 to prepare a transfer vector. Subsequently, a desired DNA fragment is inserted downstream from a polyhedrin promoter of the transfer vector, and an insect cell is concurrently infected therewith, together with baculovirus DNA, followed by cultivation. Homologous  
15 recombination is allowed to take place in the insect cell to obtain a recombinant baculovirus. The recombinant virus forms the desired product freshly introduced, instead of forming a polyhedrin. When the virus is AcNPV, a yatoga caterpillar-derived established cell line (*Spodoptera*  
20 *frugiperda* cell; Sf cell) is used as a host cell. When the virus is BmNPV, a silk worm-derived established cell line (*Bombyx mori* N; BmN cell) is used. As expression systems using baculoviruses and insect cells, commercial systems can be employed (for example, MAXBAC™, Invitrogen), and  
25 procedures described in the experimental descriptions attached thereto and in Bio/Technology, 6, 47-55 (1988) can

also be adopted. The amount and quality of the expressed receptor can be examined by methods known per se in the art, for example, the method described in P. Nambi et al. J. Biol. Chem., 267, 19555-19559 (1992).

5 In the screening methods of the present invention, as the PACAP receptor proteins, either cells containing the proteins or membrane fractions of cells containing the proteins may be used. Further, membrane fractions of insect cells containing the proteins are most preferably  
10 used.

Said cells means host cells in which the PACAP receptor proteins are expressed. Said host cells include E. coli, Bacillus subtilis, yeast, insect cells and animal cells (for example, human cells), and the insect cells are  
15 preferred among others.

The membrane fractions means fractions in which cell membranes obtained by methods known per se in the art after cell disruption are contained in large amounts.. The disruption of the cells is carried out preferably at 0 to  
20 4°C, and physiological saline or a buffer such as 50 mM Tris-HCl is used. A protease inhibitor is preferably added to prevent decomposition of the protein. Methods for disrupting the cells include the method of crushing the cells with a Potter-Elvehjem type homogenizer, disruption  
25 with a Working blender or a Polytron homogenizer (Kinematica), disruption by ultrasonication, and disruption

by allowing the cells to jet through a fine nozzle under pressing with a French press, etc. Fractionating methods utilizing centrifugal force such as differential centrifugation and density gradient centrifugation are  
5 mainly used for fractionation of the cell membranes. For example, a cell disrupted solution is centrifuged at a low speed (500 to 3000 rpm) for a short period of time (usually about 1 to 10 minutes), and the supernatant is further centrifuged at a high speed (15000 to 30000 rpm), usually  
10 for 30 minutes to 2 hours. The resulting precipitate is taken as the membrane fraction. In said membrane fraction, the expressed PACAP receptor protein and membrane compositions such as cell-derived phospholipids and membrane proteins are contained in large amounts.

15 The amount of the PACAP receptor proteins in the cells or the membrane fractions containing the PACAP receptor proteins is preferably  $10^3$  to  $10^8$  molecules per cell, and suitably  $10^5$  to  $10^7$  molecules per cell. A more expression amount results in higher PACAP binding activity per  
20 membrane fraction (specific activity). Not only construction of a high sensitive screening system becomes possible, but also a large amount of samples can be measured in the same lot.

In order to screen compounds antagonistically  
25 inhibiting the binding of a PACAP to a PACAP receptor, an appropriate PACAP receptor fraction and a labeled PACAP

(for example, PACAP27 or PACAP38, hereinafter referred to as a PACAP) are required. Desirable examples of the PACAP receptor fractions include natural PACAP receptor proteins and recombinant PACAP receptor proteins equivalent thereto.

5 As the labeled PACAPs, PACAP27 labeled with [ $^{125}\text{I}$ ] (du Pont), etc. are commercially available. They can therefore be utilized.

When the compounds antagonistically inhibiting the binding of the PACAP to the PACAP receptor is screened,  
10 cells or cell membrane fractions containing the PACAP receptor protein are first suspended in a buffer suitable for screening, thereby preparing a receptor sample. The buffer may be any, as long as it is a buffer which does not inhibit the binding of the PACAP to the receptor, such as  
15 phosphate buffer or Tris-HCl buffer having a pH of 4 to 10 (preferably a pH of 6 to 8). For the purpose of decreasing non-specific binding, a surface active agent such as CHAPS, Tween-80<sup>TM</sup> (Kao-Atlas), digitonin or deoxycholate may also be added to the buffer. Further, for the purpose of  
20 inhibiting decomposition of the receptor or a ligand with a protease, a protease inhibitor such as PMSF, leupeptin, E-67 (Peptide Laboratory) or pepstatin can also be added. A definite amount (5000 to 500000 cpm) of [ $^{125}\text{I}$ ]PACAP is added to 0.01 to 10 ml of the receptor solution, and  $10^{-4}$  to  $10^{-10}$   
25 M specimen compound, fermentation products, etc. are allowed to coexist at the same time. In order to know the

non-specific binding (NSB), a reaction tube to which a ligand is added in large excess is prepared. Reaction is conducted at 0 to 50°C, desirably at 4 to 37°C for 20 minutes to 24 hours, desirably for 30 minutes to 3 hours.

5 After reaction, the reaction product is filtered through a glass fiber filter and washed with an appropriate amount of the same buffer, followed by measurement of [ $^{125}\text{I}$ ] remaining on the glass fiber filter with a  $\gamma$ -counter. When the count ( $B_0$ -NSB) obtained by subtracting NSB from the count ( $B_0$ ) in  
10 the absence of an antagonistic substance is taken as 100%, the specimen compound, the fermentation products, etc giving a non-specific binding (B-NSB) of 50% or less can be selected as potential materials having antagonistic ability.

15 Examples of kits for screening the compounds antagonistically inhibiting the binding of the PACAPs to the PACAP receptors of the present invention include the following:

1. Reagents for Screening

20 (A) Buffer for Measurement

	Tris-HCl	2.4 g
	Magnesium acetate·4H <sub>2</sub> O	1.07 g
	EGTA	0.76 g
	NaN <sub>3</sub>	0.6 g
25	Leupeptin	20 mg
	E-64	4 mg

These are dissolved in 997 ml of distilled water.

Pepstatin	1 mg
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PMSF	0.09 g
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These are dissolved in 1 ml of DMSO, and the resulting  
5 solution is added to 997 ml of the above-mentioned water.  
About 2 ml of 6 N HCl is added thereto to adjust to pH 7.2.  
One gram of BSA is dissolved therein, followed by storage  
at 4°C.

(B) Buffer for Washing

10 CHAPS	0.45 g
----------	--------

This is dissolved in 900 ml of the buffer for  
measurement and the solution is stored at 4°C.

(C) PACAP Receptor Sample

15 A membrane fraction of insect cells (Sf9) in which a  
PACAP receptor protein is expressed is diluted with the  
buffer for measurement to 0.5 to 5 µg of protein/ml before  
use.

(D) [<sup>125</sup>I] Labeled PACAP

(3-[<sup>125</sup>I]iodotyrosyl)

20 PACAP (du Pont)	185 kBq
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Fifty microliters of distilled water is added thereto  
to dissolve it, and 450 µl of the buffer for measurement is  
added thereto. The solution is stored at -20°C.

(E) PACAP Standard Solution

25 The PACAP (Peptide Laboratory) is diluted with 50%  
DMSO to 10<sup>-4</sup> M, and stored at -20°C. This is diluted 10



times with the buffer for measurement before use.

## 2. Assays

(i) The membrane fraction of Sf9 cells containing the PACAP receptor protein [J. L. Vaughn et al., In Vitro, 13, 213-217 (1977)] is diluted with the buffer for measurement to give 1 µg of protein/ml, and 100 µl thereof is poured into each tube (Falcon).

(ii) After addition of 3 µl of  $10^{-4}$  to  $10^{-10}$  M specimen or 10µl or less of fermentation products, 2 µl of [ $^{125}$ I] labeled PACAP is added, followed by reaction at 25°C for 60 minutes. In order to examine the non-specific bonding, 3 µl of  $10^{-5}$  M PACAP is added instead of the specimen.

(iii) The buffer for washing (1.5 ml) is added, and filtration is conducted through a glass fiber filter (GF/F, Whatman). Then, 1.5 ml of the same buffer is further added to the residue in the tube, and filtration is conducted again.

(iv) [ $^{125}$ I] remaining on the glass fiber filter is measured with a γ-counter, and the percent maximum binding (PMB) is determined from the following equation;

$$\text{PMB} = [(B - \text{NBS}) / (B_0 - \text{NBS})] \times 100$$

PMB: percent maximum binding

B: value when the specimen is added

NBS: non-specific binding

25 B<sub>0</sub>: maximum binding

(II) Methods for Screening Compounds Activating the

## PACAP Receptors

The compounds antagonistically inhibiting the binding of the PACAPs to the PACAP receptor proteins selected by the methods of (I) described above is expected to contain  
5 compounds activating the PACAP receptor proteins similarly to the PACAPs (compounds having PACAP receptor agonist activity). Such compounds can be evaluated by secondary screening systems based on acceleration of cyclic AMP production as described below.

10 First, cells in which the PACAP receptor protein is expressed are subcultured to a 48-well plate for tissue culture in a ratio of  $1 \times 10^5$  cells/well, and cultured for 2 days. Then, the medium is removed, and the plate is washed twice with serum-free medium. Subsequently, 300  $\mu$ l  
15 of the same medium is added to each well as a reaction solution. The serum-free medium may be any, as long as it is a medium for cell culture, and bovine serum albumin, etc. may be added for the purpose of preventing the compounds added from being non-specifically adsorbed by the  
20 instruments, etc. Further, for the purpose of inhibiting decomposition of cyclic AMP produced to enhance assay sensitivity, addition of 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, is effective. The specimen compound having a final concentration of  $10^{-4}$  to  
25  $10^{-10}$  M and fermentation products are added to each well. In order to know non-specific response, wells containing

only the solvent in which the compounds are dissolved are prepared. Reaction is usually conducted at 4 to 42°C for 10 minutes to 2 hours, preferably at room temperature to 37°C for 20 minutes to 1 hour. After reaction, the

5 supernatant is removed by suction. After washing with two portions of the reaction solution, cyclic AMP produced is extracted with 200 µl of 100% ethanol. Ethanol is removed with a centrifugal freeze dryer, and the residue is dissolved in 100 µl of a buffer for determination of cyclic  
10 AMP. Reagents for determination of cyclic AMP, including the buffer, may be ones commercially available as kits according to either radio immunoassay (RIA) or enzyme immunoassay (EIA) (Amersham, du Pont, etc.). When the production amount of cyclic AMP which has become clear by  
15 determination is statistically significantly high, compared with the case where the sample is not added or the case where only the solvent in which the sample is dissolved is added, such compound can be selected as potential compounds having PACAP receptor agonist activity. In order to  
20 eliminate the probability that the cyclic AMP production promoting action of the potential compounds is non-specific action to cells or action through receptors other than the PACAP receptor, it is necessary to confirm that the potential compounds exhibit no cyclic AMP production  
25 promoting action in cells in which the PACAP receptor protein is not allowed to be expressed. As an indication

for PACAP receptor agonist activity, production promotion of inositol triphosphate or diacylglycerol and an increase in intracellular calcium concentration, as well as the production promotion of cyclic AMP, may be employed.

5 However, the production promotion of cyclic AMP is superior from the viewpoint of treating the sample in large amounts. Such screening methods of the present invention are excellent methods by which compounds having action similar to that of the PACAP or higher than the PACAP and excellent  
10 in resistance against proteases, compared with the PACAP, a peptide, can be selected.

Antibodies or antiserum to the PACAP receptor proteins of the present invention, the partial peptides thereof or the salts thereof may be any antibodies or antiserum as  
15 long as they can recognize the PACAP receptor proteins, the partial peptides thereof or the salts thereof. For example, monoclonal antibodies such as PRN1-25a, PRN1-109a and PRN1-159a against a partial peptide (MHSDAIFKKEQAMC)  
are preferable. The partial peptide was prepared by  
20 substituting the 5th Cys(C) of a partial peptide which has a partial amino acid sequence (1st to 14th amino acid sequence of SEQ ID NO:14) common to bovine, rat or human PACAP receptor which has amino acid sequence of anyone of  
SEQ ID NO:14 to SEQ ID NO:29 to Ala(A), for the convenience  
25 of preparation of immunoantigen complexes.

Antibodies or antiserum to the PACAP receptor proteins

5 of the present invention, the partial peptides thereof or  
the salts thereof can be produced by methods known per se  
in the art, using the PACAP receptor proteins, the partial  
peptides thereof or the salts thereof as antigens. The  
antibodies or antiserum thus obtained can be used for  
quantitative analysis or detection of the PACAP receptor  
10 proteins of the present invention, the peptides thereof or  
the salts thereof, more detailed utilities are as follows:

15 (1) By using the antibodies or antiserum for Western  
blotting or immune precipitation, the PACAP receptor  
proteins, the partial peptides thereof or the salts thereof  
can be detected.

(2) An affinity column to which the antibodies of the  
present invention are fixed, can purify the PACAP receptor  
proteins, the partial peptides thereof or the salts  
thereof.

20 (3) The antibodies of the present invention can be  
used as a PACAP receptor antagonist, as shown in Example  
12, since the antibodies block PACAP action by inhibiting  
binding of PACAP and a PACAP receptor.

25 As a signal peptide of the PACAP receptor protein of the  
present invention, for example, a peptide which has 1st to 37th  
amino acid sequence of SEQ ID NO:15, a peptide which has 1st to  
37th amino acid sequence of SEQ ID NO:17, a peptide which has  
1st to 19th amino acid sequence of SEQ ID NO:19, a peptide  
which has 1st to 19th amino acid sequence of SEQ ID NO:21,  
30

a peptide which has 1st to 77th amino acid sequence of SEQ ID NO:23, a peptide which has 1st to 77th amino acid sequence of SEQ ID NO:25, a peptide which has 1st to 77th amino acid sequence of SEQ ID NO:27, a peptide which has 1st to 77th amino acid sequence of SEQ ID NO:29, a peptide which has 58th to 77th amino acid sequence of SEQ ID NO:23, a peptide which has 58th to 77th amino acid sequence of SEQ ID NO:25, a peptide which has 58th to 77th amino acid sequence of SEQ ID NO:27 or a peptide which has 58th to 77th amino acid sequence of SEQ ID NO:29 may be used.

These signal peptides can be synthesized by conventional methods such as a peptide synthesizer or prepared by cutting the amino acid bond of the PACAP receptor of the present invention with an enzyme.

The salts of the signal peptides of the present invention include similar salts as those for PACAP receptors or partial peptides thereof.

A DNA which encodes a signal peptide may be any one which has a nucleotide sequence encoding the signal peptide and includes a DNA which has 1st to 111th nucleotide sequence of SEQ ID NO:30, a DNA which has 1st to 111th nucleotide sequence of SEQ ID NO:31, a DNA which has 1st to 57th nucleotide sequence of SEQ ID NO:32, a DNA which has 1st to 57th nucleotide sequence of SEQ ID NO:33, a DNA which has 1st to 231st nucleotide sequence of SEQ ID NO:34, a DNA which has 1st to 231st nucleotide sequence of SEQ ID

5 NO:35, a DNA which has 1st to 231st nucleotide sequence of  
SEQ ID NO:36, a DNA which has 1st to 231st nucleotide  
sequence of SEQ ID NO:37, a DNA which has 172nd to 231st  
nucleotide sequence of SEQ ID NO:34, a DNA which has 172nd  
to 231st nucleotide sequence of SEQ ID NO:35, a DNA which  
has 172nd to 231st nucleotide sequence of SEQ ID NO:36, a  
10 DNA which has 172nd to 231st nucleotide sequence of SEQ ID  
NO:37 or a DNA which comprises one of these DNAs. These  
DNAs encoding signal peptides of the present invention can  
be synthesized by conventional method such as a peptide  
synthesizer or prepared by cutting the DNA (cDNA is  
15 preferable) which encodes the PACAP receptor of the present  
invention with an appropriate restrictive enzyme.

The DNA coding for the signal peptide of the PACAP  
receptor proteins of the present invention may stimulate an  
expression of a membrane-bound peptide such as a receptor  
into a membrane. For example, a protein which does not or  
20 rarely expresses into a membrane can be expressed on the  
membrane effectively by linking a DNA coding for a signal  
peptide of the PACAP receptor proteins of the present  
invention upstream from the DNA which rarely or does not  
25 express the desired protein on the membrane in an  
expression.

The present invention will be described in more detail  
through the following examples. It is understood of course  
that they are not intended to limit the scope of the  
30 invention.

Transformant E. coli pBPR-T containing pBPRT and

transformant E. coli pBPR114 containing pBPR114 each  
obtained in Example 2 given later were deposited with the  
National Institute of Bioscience and Human-technology  
(NIBH), the Agency of Industrial Science and Technology,  
5 the Ministry of International Trade and Industry, Japan,  
under the accession numbers FERM BP-4338 and FERM BP-4339,  
respectively, on June 15, 1993, and deposited with  
Institute for Fermentation, Osaka, Japan (IFO) under the  
accession numbers IFO 15572 and IFO 15571, respectively, on  
10 November 5, 1993.

Transformant E. coli pRPACAPR 12 containing pRPACAPR  
12 and transformant E. coli pRPACAPR 46-5 containing  
pRPACAPR each obtained in Example 3 given later were  
deposited with NIBH, under the accession numbers FERM BP-  
15 4254 and FERM BP-4255, respectively, on April 5, 1993, and  
deposited with IFO under the accession numbers IFO 15469  
and IFO 15470, respectively, on April 15, 1993.

Transformant E. coli MV1184/pTS847-1 containing  
pTS847-1 obtained in Example 4 given below was deposited  
20 with the NIBH under the accession number FERM BP-4280, and  
deposited with IFO under the accession number IFO 15570 on  
November 5, 1993.

Transformant E. coli pHPR15A containing pHPR15A  
obtained in Example 4 given below; Transformant E. coli  
25 pHPR55A containing pHPR55A and Transformant E. coli pHPR66P  
containing pHPR66P were deposited with the NIBH under the



accession number FERM BP-4511, FERM BP-4510 and FERM BP-4509, respectively on December 22, 1993, and deposited with IFO under the accession number IFO 15603, 15604 and 15605, respectively on December 20, 1993.

- 5       Hybridoma PRN1-159 obtained in Example 12 given below was deposited with NIBH under the accession number FERM BP-4554 on February 8, 1994, and deposited with IFO under the accession number IFO 50427 on February 8, 1994.

#### EXAMPLES

- 10      [Example 1] Production (Purification) of Bovine-Derived PACAP Receptor Protein

The following procedure was conducted in a low temperature laboratory at 4°C.

##### (1) Preparation of Membrane Fractions

- 15       Membrane fractions were prepared from the bovine cerebrums according to a method in which the known method described in Biochem. Biophys. Res. Commun., 172, 709-714 (1990) was partially modified. The fresh bovine cerebrums (1.5 kg) were homogenized 3 times in 6 liters of buffer A
- 20      (20 mM Tris, 10 mM EDTA, 0.25 M sucrose, 0.5 mM PMSF, 20 µg/ml leupeptin, 4 µg/ml E-64 and 1 µg/ml pepstatin, pH 7.4) with a Polytron homogenizer (Kinematica) for 30 seconds. The resulting homogenate was centrifuged with a high speed cooling centrifuge CR26H, Roter RR10A (Hitachi,
- 25      Ltd.) at 680 X g for 20 minutes to obtain a supernatant. The resulting supernatant was further ultracentrifuged with

an ultracentrifuge SCP70H, Roter RPZ35T (Hitachi, Ltd.) at 100,000 X g for 60 minutes to obtain pellets. The pellets were suspended in 400 ml of buffer B (20 mM Tris, 5 mM EDTA, 0.5 mM PMSF, 20 µg/ml leupeptin, 4 µg/ml E-64 and 1 µg/ml pepstatin, pH 7.4) to prepare a membrane fraction suspension.

### (2) Solubilization of the PACAP Receptor Protein from the Membrane Fractions

The membrane fraction suspension obtained in (1) described above (400 ml) was diluted with 5 liters of buffer B to give a membrane protein concentration of 1 mg/ml, and digitonin was added thereto to provide a concentration of 1%. The resulting suspension was slowly stirred for 1 hour, and then, ultracentrifuged with an ultracentrifuge SCP70H, Roter RPZ35T (Hitachi, Ltd.) at 100,000 X g for 1 hour to obtain a supernatant. The resulting supernatant was used as a solubilized membrane protein fraction.

### (3) Assay of Receptor Activity of the PACAP Receptor Protein

PACAP receptor activity was assayed according to the saturation binding experiment method using [<sup>125</sup>I]PACAP27 and the antagonistic binding experiment method [Biochem. Biophys. Res. Commun., 171, 838-844 (1990) and Biochem. Biophys. Res. Commun., 172, 709-714 (1990)]. The test sample (membrane fraction or solubilized membrane protein

fraction) was appropriately diluted with buffer D (20 mM Tris, 5 mM magnesium chloride, 0.1% BSA and 0.05% digitonin, pH 7.4). In the saturation binding experiment, 0.1 ml of the diluted test sample was mixed with 10  $\mu$ l of

5  $[^{125}\text{I}]\text{PACAP27}$  (final concentration: 20 to 50 pM), and reacted at 25°C for 1 hour. In the competitive binding experiment, the diluted test sample was mixed with 2  $\mu$ l of  $[^{125}\text{I}]\text{PACAP27}$  (final concentration: 100 pM) and 3  $\mu$ l of an unlabeled peptide (PACAP27, PACAP38 or VIP) having a

10 variable concentration, and reacted in a similar manner. To 0.1 ml of this reaction solution, 1.5 ml of buffer E (0.1% BSA, 0.05% CHAPS, 20 mM Tris and 5 mM magnesium chloride, pH 7.4) cooled with ice was added, and immediately, the mixed solution is filtered through a glass

15 fiber filter. The glass fiber filter used had previously been treated with 0.3% polyethylene imine. The radioactivity of the filter was counted with a  $\gamma$ -ray counter, thereby determining  $[^{125}\text{I}]\text{PACAP27}$  bound to the receptor. In order to determine the non-specific binding,

20 the above-mentioned experiment was carried out in the presence of 1  $\mu$ M PACAP27. The specific binding was calculated by subtracting the non-specific binding from the total binding measured in the absence of PACAP27. Results of the saturation binding experiment were subjected to

25 Scatchard plot analysis to determine the dissociation constant and the maximum binding.

#### (4) Crude Purification of the PACAP Receptor Protein

A method for purifying the PACAP receptor from the solubilized membrane protein fraction by ion exchange chromatography and hydroxyapatite chromatography is

5 described below.

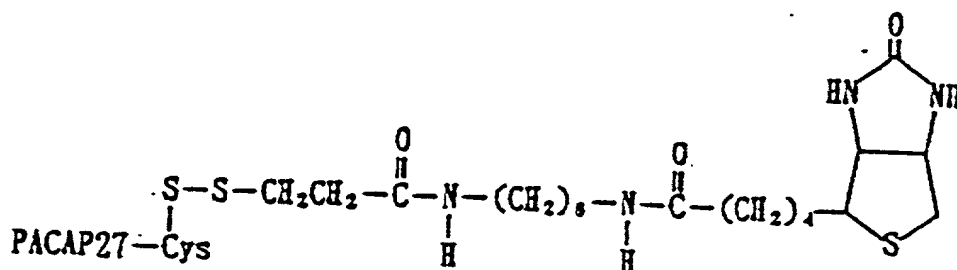
The solubilized membrane protein fraction [2400 mg (4800 ml)] was loaded onto an ion exchange column (for example, anion exchange chromatography such as DEAE-TOYOPEARL) equilibrated with 1 liter of buffer C, at a flow  
10 rate of 9 ml/minute. Then, the concentration of sodium chloride in buffer C (20 mM Tris, 1 mM EDTA, 0.5 mM PMSF, 20 µg/ml leupeptin, 4 µg/ml E-64 and 1 µg/ml pepstatin, pH 7.4) supplemented with 0.1% digitonin was gradually increased from 0 M to 1 M for 170 minutes to elute the  
15 PACAP receptor from the column. The PACAP receptor activity of each eluted fraction was assayed by the above-mentioned method. The active fractions eluted from the ion exchange column were further loaded onto a hydroxyapatite column (HCA-100, 5 cm in diameter and 7 cm in length) at a  
20 flow rate of 7 ml/minute. This column was washed with 500 ml of 0.1 M phosphate buffer containing 0.1% digitonin, and then, the PACAP receptor was eluted with 500 ml of 0.6 M phosphate buffer containing 0.1% digitonin at a flow rate of 7 ml/minute. The active fractions were concentrated 10-  
25 fold using an ultrafilter, and further desalted by repetition of dilution and concentration with a 6-fold

excess of buffer C in relation to the volume of the concentrated sample.

# (5) Purification of the PACAP Receptor by Affinity Chromatography

## 5 (5-1) Preparation of Affinity Ligand

A method for preparing a biotininated PACAP used in affinity chromatography is described below. One equivalent of the PACAP27 derivative (having cysteine as the 28th amino acid residue, PACAP27-Cys) synthesized by the solid phase method was dissolved in 50 mM phosphate buffer (pH 7.0) supplemented with 3 mM EDTA and 0.5 M NaCl to provide a concentration of  $2 \times 10^{-4}$ , and a 10 mM biotinylating reagent (biotin-HSDP) dissolved in DMF was added thereto to give 10 equivalents,\* followed by reaction overnight. The reaction product, biotinylated PACAP27 (PACAP27-Cys-biotin) represented by the following formula, was purified on a reverse phase HPL chromatography:



Namely, the reaction product was loaded onto a reverse phase column (ODS 80-TM, Tosoh) equilibrated with 60 ml of distilled water containing 0.05% TFA, and the concentration of acetonitrile was gradually increased from 20% to 40% for

60 minutes at a flow rate of 1 ml/minute at room temperature to conduct separation. Peak fractions of biotinylated PACAP27 were fractionated, and chromatographed again under the same conditions (Fig. 28) to obtain pure biotinylated PACAP27, followed by lyophilization. It was confirmed by the competitive binding experiment that biotinylated PACAP27 has an affinity similar to that of PACAP (Fig. 29).

#### (5-2) Affinity Chromatography

Avidin-agarose was suspended in a solution containing the PACAP receptor protein crudely purified by the method described above, and gently stirred overnight. Avidin-agarose was removed by filtration to obtain a filtrate. About 20-fold equivalents of biotinylated PACAP27 in relation to the amount of the receptor was added to this filtrate, and allowed to react overnight. Further, 80 ml of avidin-agarose was suspended therein, and gently stirred for 4 days. This avidin-agarose was packed into a column, and washed with 500 ml of buffer C containing 1 M sodium chloride and 0.1% digitonin at a flow rate of 1.5 ml/minute, followed by elution of the PACAP receptor protein with 180 ml of a buffer (20 mM magnesium acetate 1 M sodium chloride and 10% glycerol, pH 4.0) at a flow rate of 1.5 ml/minute. The eluate was immediately neutralized with 1/4 volume of 1 M Tris (pH 7.5) with respect to the eluate.

(6) Final Purification after Affinity Chromatography

The PACAP receptor protein purified by the above-mentioned affinity chromatography was loaded onto a microcolumn (1.8 ml) of hydroxyapatite at a flow rate of 0.3 ml/minute, and washed with 20 ml of 0.1 M phosphate buffer containing 0.1% digitonin at a flow rate of 0.3 ml/minute, followed by elution of the PACAP receptor from the column with 20 ml of 0.6 M phosphate buffer containing 0.1% digitonin at a flow rate of 0.3 ml/minute. The active fractions eluted were concentrated using an ultrafilter (CENTRICON 10<sup>TM</sup>, Amicon). The active fractions concentrated were gel filtered on a gel filtration column (for example, Superrose 6 Column, Pharmacia) equilibrated with 60 ml of buffer C containing 0.1% digitonin and 0.2 M NaCl at a flow rate of 0.4 ml/minute. The active fractions eluted were used as a purified PACAP receptor protein sample.

One embodiment of the purification procedure conducted by the above-mentioned methods is summarized in Table 2.

The specific activity (mole number of PACAP binding to unit weight of protein) of the final purified sample determined by the saturation binding experiment using [<sup>125</sup>I]PACAP27 was usually 15,000 pmoles/mg of protein or more. Further, the calculation of the dissociation constant from results of the saturation binding experiment revealed that the dissociation constant of the final

purified sample approximately agrees with that of the PACAP  
receptor existing in the membrane fractions, and that the  
purified PACAP receptor protein has a sufficiently high  
affinity for the PACAP (Fig. 30). Furthermore, results of  
5 the competitive binding experiment for the purified PACAP  
receptor protein proved that it has the property of  
reacting with PACAP27 and PACAP38, but not reacting with  
VIP (Fig. 31). Analysis results obtained by polyacrylamide  
electrophoresis for the final purified sample in the  
10 presence of sodium dodecylsulfate are shown in Fig. 28.  
The results indicate that the final purified sample is  
composed of a substantially pure protein (molecular weight:  
about 57,000). This protein having a molecular weight of  
about 57,000 is the PACAP receptor protein occurring in the  
15 bovine cerebrums.



[TABLE 2]

	Total activity (pmole)	Total protein (mg)	Specific activity (pmole/mg)	Purification (fold)	Activity yield (%)
5					
	8115	6400	1.3		
	4561	2400	1.9	1	100
10	4700	475	9.9	5.2	103
	3349	134	25.0	13.2	73
	2040	ND	ND		45
15	1717	ND	ND		38
	671	0.042	16000	8400	15

20 Total activity: The maximum binding of [ $^{125}$ I]PACAP27 obtained by the saturation binding experiment

ND: Not determined

[Example 2] Screening of Bovine PACAP Receptor Protein cDNA  
and DNA Sequence Analysis

(1) Preparation of Bovine Hippocampus Poly(A)<sup>+</sup> RNA  
Fractions and Construction of cDNA Library Using the Same

5        Total RNA fractions were prepared from the bovine  
hippocampi according to the guanidine-fluoroacetate method  
[Method in Enzymology, 154, 3 (1987) and Biochemistry, 18,  
5294 (1978)], and poly(A)<sup>+</sup> RNA fractions were further  
separated by the use of an oligo(dT) cellulose-spun-column  
10 (Pharmacia). Using these fractions as a starting material,  
a bovine hippocampus cDNA library in which a vector was  
λgt11 was constructed by the use of a cDNA cloning kit  
(Amersham). The library prepared had about 4X10<sup>6</sup> pfu  
(plaque forming unit) of independent clones.

15        (2) Preparation of Probe

A synthetic DNA was prepared as a probe, based on the  
N-terminal amino acid sequence having the amino acid  
sequence represented by SEQ ID NO: 38 of the bovine PACAP  
receptor protein obtained in Example 1.

20        Sequence:

5' TGGATCTTCTCCAGGTGCATDGCCTGCTCCTTCTTGAAGATGTGGTC 3'  
(SEQ ID NO: 51)

(D is G, A or T.)

(3) Screening

25        The λgt11 phage cDNA library (bovine brain, Clontech)  
(1.5X10<sup>6</sup> pfu) prepared in Example 2 (1) was mixed with  
magnesium sulfate-treated E. coli Y1090, and incubated at

37°C for 15 minutes. Then, 0.5% agarose/LB was added thereto, followed by plating on a 1.5% agar/LB plate. A nitrocellulose filter was placed on the plate on which a plaque is formed, and the plaque transferred onto the  
5 filter. After alkali treatment of this filter, the DNA was fixed by heating at 80°C for 3 hours. This filter was hybridized with the labeled probe in a hybridization buffer [0.5 M phosphate buffer (pH 7.2), 1% bovine serum albumin, 7% SDS and 1 mM EDTA] overnight at 50°C. The labeling of  
10 the probe was conducted according to the method of phosphorylation of the 5'-terminus of the probe with [ $\gamma$ -<sup>32</sup>P]ATP and T4 polynucleotide kinase (Nippon Gene). Washing was carried out with 2 X SSC, 0.1% SDS at 48°C for 1 hour, and then, hybridized clones were detected by  
15 autoradiography at -80°C. As a result, a cDNA encoding a N-terminal portion of the PACAP receptor was obtained, and the cDNA designated as  $\lambda$ BPR35.

Further, the bovine brain-derived cDNA library (Clontech) ( $1.5 \times 10^6$  pfu) was screened, using the cDNA  
20 portion of  $\lambda$ BPR35 as a probe, to obtain a cDNA encoding C-terminal portion of the PACAP receptor. At this time, a buffer was used which comprised 5 X Denhardt's solution [0.02% bovine serum albumin (Sigma)], 5 X SSPE (0.15 M sodium chloride, 0.01 M monosodium phosphate and 1 mM  
25 EDTA), 0.1% SDS and 100  $\mu$ g/ml of heat-denatured salmon sperm DNA (Sigma), and incubation was conducted overnight at 65°C together with the labeled probe to hybridize. The

labeling of the probe was carried out by the use of a multi-prime DNA labeling kit (Amersham). Washing was carried out with 0.2 X SSC, 0.1% SDS at 60°C for 1 hour, and then, hybridized clones were detected by

5 autoradiography at -80°C. A cDNA clone which encoded a portion of the PACAP receptor was obtained, and the cDNA was designated as  $\lambda$ BPR114. Using the cDNA portion of the resulting pBPR114 as a probe, the cDNA library ( $4 \times 10^{-6}$  pfu) prepared from the bovine hippocampus poly(A)<sup>+</sup> RNA fractions  
10 was screened to obtain a cDNA encoding the C-terminal portion of the PACAP receptor. The conditions at this time were the same as those at the time when the above-mentioned  $\lambda$ BPR114 was screened. As a result, a cDNA clone encoding a C-terminal portion of the PACAP receptor was obtained, and  
15 the cDNA was designated as  $\lambda$ BPR68.

#### (4) Subcloning of cDNA Clones and DNA Sequence Analysis

An inserting portion of the resulting cDNA clone was cut out by cleavage with EcoRI, and subcloned into plasmid  
20 vector pUC118 to obtain pBPR35, pBPR114 or pBPR68. The plasmid was further cleaved stepwise by exonuclease digestion, or self cyclized or subcloned after cleavage with an appropriate restriction enzyme (NcoI, BamHI, etc.) to prepare a template DNA for sequence analysis. For  
25 sequence determination, the dideoxy chain termination method using RI marker dCTP and a fluorescent DNA sequencer (Applied Biosystems) were used, and for data analysis, a

DNASIS (Hitachi Software Engineering) was used. Further, pBPR35 and pBPR68 were recombined at the BamHI sites to prepare pBPR-T. The BamHI and AvalI fragments of pBPR114 having disappeared regions can be recombined with pBPR-T by the use of known genetic engineering technique, thereby preparing PACAP receptor cDNA (pBPR-TD) containing no insertion.

Results of analysis revealed that pBPR-T has the nucleotide sequence of SEQ ID NO: 38, and that pBPR-TD has the nucleotide sequence of SEQ ID NO: 39.

#### [Example 3] Screening of Rat PACAP Receptor Protein cDNA and DNA Sequence Analysis

##### (1) Preparation of Rat Brain Poly(A)<sup>+</sup> RNA Fractions and Construction of cDNA Library Using the Same

Total RNA fractions were prepared from the rat brains according to the guanidine-isothiocyanate method [Biochemistry, 18, 5294 (1979)], and poly(A)<sup>+</sup> RNA fractions were further separated by the use of an oligo(dT) cellulose-spun-column (Pharmacia). Using these fractions as a starting material, a rat brain cDNA library in which a vector was  $\lambda$ gt11 was constructed by the use of a cDNA cloning kit (Amersham). The library prepared had about  $3 \times 10^6$  pfu (plaque forming unit) of independent clones.

##### (2) Preparation of Probe

Based on the cDNA nucleotide sequence of rat VIP receptor already reported, primers for PCR were synthesized with a DNA synthesizer (Model 391, PCR-MATE EP, Applied

Biosystems).

Sequence: RVIPLR-1S

5' CAGA AAGCTT CGGACCATGCGCCCTCCGAGCCCACCG 3'

(SEQ ID NO: 48)

5 Sequence: RVIPLR-2A

5' GGGC TCTAGA CGGTCAGACCAGGGAGACCTCCGCTTG 3'

(SEQ ID NO: 49)

Using 5 µg of rat lung poly(A)<sup>+</sup> RNA fractions prepared in a manner similar to that of the brain RNA fractions and a random primer, cDNA having only first strand was synthesized. Then, using this single stranded DNA as a template, and using the above-mentioned primers, rat VIP receptor cDNA fragments were amplified by the PCR method. The sequences of the resulting cDNA fragments were determined, and they are confirmed to be cDNA fragments of rat VIP receptor.

### (3) Screening

The λgt11 cDNA library (3X10<sup>6</sup> pfu) prepared in Example 3 (1) was mixed with magnesium sulfate-treated E. coli Y1090, and incubated at 37°C for 15 minutes. Then, 0.5% agarose/LB was added thereto, followed by plating on a 1.5% agar/LB plate. A nitrocellulose filter was placed on the plate on which a plaque is formed, and the plaque was transferred onto the filter. After alkali treatment of this filter, the DNA was fixed by heating at 80°C for 3 hours. This filter was hybridized with the probe labeled in hybridbuffer S [0.2% poly(vinylpyrrolidone), 0.2% bovine

serum albumin, 0.2% ficoll 400, 2 X SSC and 0.17% yeast RNA) overnight at 55°C. The labeling of the probe was conducted by the use of a multi-prime labeling kit (Amersham). Washing was carried out with 2 X SSC, 0.1% SDS at 50°C for 1 hour, and then, hybridized clones were detected by autoradiography at -80°C. As a result,  $\lambda$ RPACAPR18 was obtained.

Further, the rat brain-derived 5'-extended cDNA library (Clontech) ( $1.7 \times 10^6$  pfu) was screened, using the cDNA portion of  $\lambda$ RPACAPR18 as a probe, to obtain  $\lambda$ RPACAPR46,  $\lambda$ RPACAPR5,  $\lambda$ RPACAPR12, etc. At this time, a buffer was used which comprised 50% formamide (Bethesda Research Laboratories), 5 X Denhardt's solution [0.02% bovine serum albumin (Sigma)], 0.02% poly(vinylpyrrolidone) (Sigma), 0.02% ficoll (Sigma), 5 X SSPE (0.15 M sodium chloride, 0.01 M monosodium phosphate and 1 mM EDTA), 0.1% SDS and 100  $\mu$ g/ml of heat-denatured salmon sperm DNA (Sigma), and incubation was conducted overnight at 42°C together with the labeled probe to hybridize. Washing was carried out with 2 X SSC, 0.1% SDS at 55°C for 1 hour, and then, hybridized clones were detected by autoradiography at -80°C.

#### (4) Subcloning of cDNA Clones and DNA Sequence Analysis

An insert portion of the resulting cDNA clone was cut out by cleavage with EcoRI, and subcloned into plasmid vector pCDNAI or pUC118 to obtain pRPACAPR18 (pCDNAI),

5 pRPACAPR46 (pcDNAI), pRPACAPR5 (pcDNAI) or pRPACAPR12 (pUC118). Further, pRPACAPR46 and pRPACAPR5 were recombined at the BamHI sites to prepare pRPACAPR46-5. The plasmid was further cleaved stepwise by exonuclease digestion, or self cyclized or subcloned after cleavage with an appropriate restriction enzyme (NcoI, PstI or BamHI) to prepare a template DNA for sequence analysis. For sequence determination, a fluorescent DNA sequencer (Applied Biosystems) was used, and for data analysis, a DNASIS (Hitachi Software Engineering) was used. Results of analysis revealed that pRPACAPR46-5 has the nucleotide sequence of SEQ ID NO: 40, and that pRPACAPR12 has the nucleotide sequence of SEQ ID NO: 41.

[Example 4] Screening of Human PACAP Receptor Protein cDNA and DNA Sequence Analysis

(1) Preparation of Probe

The nucleotide sequence represented by SEQ ID NO: 51 corresponding to a complementary strand of the N-terminal amino acid sequence having the amino acid sequence represented by SEQ ID NO: 50 of the bovine PACAP receptor protein obtained in Example 1 was synthesized with a DNA synthesizer (Model 391, PCR-MATE EP, Applied Biosystems).

(2) Screening

The human pituitary gland-derived cDNA library ( $\lambda$ gt11, Clontech) ( $1.4 \times 10^6$  pfu) was mixed with magnesium sulfate-treated E. coli Y1090, and incubated at 37°C for 30 minutes. Then, 0.6% agarose/LB was added thereto, followed



by plating on a 1.5% agar/LB + 50 µg/ml ampicillin plate. A nitrocellulose filter was placed on the plate on which a plaque is produced, and the plaque was transferred onto the filter. After alkali treatment and neutralization

5 treatment of this filter, the DNA was fixed by heating at 80°C for 3 hours. This filter was prehybridized in a hybridization buffer [7% SDS (Nakarai), 1% bovine serum albumin, 0.5 M Na-PO<sub>4</sub> (pH 7.2) and 1 mM EDTA (Dojin)], and then hybridized with the probe labeled in the same buffer  
10 overnight at 55°C. For the labeling of the probe, terminal labeling was conducted using [ $\gamma$ -<sup>32</sup>P]ATP (Du Pont NEN) and T4 kinase (Takara). Washing was carried out twice with 2 X SSC, 0.1% SDS at 55°C for 30 minutes, and then, hybridized clones were detected by autoradiography at -80°C. As a  
15 result, λ#14 was obtained.

### (3) Subcloning of cDNA Clones and DNA Sequence Analysis

An insert portion of the resulting cDNA clone was cut out by cleavage with EcoRI, and subcloned into plasmid  
20 vector pUC118 to obtain pTS847-1. After further cleavage with an appropriate restriction enzyme (SacI, NcoI or HpaI), the plasmid was self cyclized to prepare a template DNA for sequence analysis. For sequence determination, a Bca Best Sequencing Kit (Takara) was used, and for data  
25 analysis, a DNASIS (Hitachi Software Engineering) was used. Results of analysis revealed that pTS847-1 has the nucleotide sequence of SEQ ID NO: 42. Among the nucleotide

sequences, the nucleotide sequence coding for mature human PACAP receptor Type I-A is represented by SEQ ID NO:34. The deduced amino acid sequence of human PACAP receptor Type I-A is represented by SEQ ID NO:23.

- 5 (4) Preparation of a primer for PCR based on the nucleotide sequence of human PACAP receptor Type I-A

A region into which the insertion region of human PACAP receptor being deduced to enter was amplified by PCR. Primers of following SEQ ID NO:52 and SEQ ID NO:53 were prepared based on the nucleotide sequence of pTS847 coding for human PACAP receptor Type I-A obtained in Example 4(3).

Sequence:HPRF

5'CTGGGATATGAATGACAGCACAGC 3' (SEQ ID NO:52; a nucleotide sequence of 1132nd to 1155th of pTS847)

- 15 Sequence:HPRR

5'TCTGGGGAGAAGGCAAATACTGTG 3' (SEQ ID NO:53; a complementary nucleotide sequence of 1342nd to 1355th of pTS847)

- 20 (5) Application of PCR on human pituitary and amigdaloid nucleus

Two(2) ng of cDNA of human pituitary and amigdaloid nucleus (Quick-Clone cDNA, Clonetech) and each 0.5 $\mu$ M of primers obtained Example 4(4), each 10 mM of dNTP were mixed in a PCR reaction buffer, and Taq polymerase was added thereto. Denaturing was conducted at 94°C for 45 seconds, anealing was held at 60°C for 45 seconds and elongation reaction was held at 72°C for 2.5 minutes to

obtain PCR product.

(6) Subcloning of PCR product and DNA sequence analysis

The resulting PCR product was inserted into Hinc II site of a plasmid pUC118 and was subjected to a subcloning.

5 Of the clones subcloned, Southern blotting was conducted to screen subtypes. In order to screen a clone of human PACAP receptor Type I-B, the following probe of SEQ ID NO:54 was synthesized based on the sequence of the insertion region of rat PACAP receptor Type I-B.

10 5'TGCGTGCAGAAATGCTACTGCAAGCCACAG 3' (SEQ ID NO:54)

In order to screen a clone of human PACAP receptor Type I-C, the following probe of SEQ ID NO:54 was synthesized based on the sequence of the insertion region which is different from Type I-B which was reported in rat  
15 (Nature, 365, p170-175, 1993).

5'GACCCCCTGCCTGTGCCCTCAGACCAGCAT 3' (SEQ ID NO:55)

Clones of pHPR15A and pHPR55A were obtained from the Southern blot of SEQ ID NO:54 and a clone of pHRP66P was obtained from the Southern blot of SEQ ID NO:55.(Fig. 15).

20 Dideoxy method using RI labelled dCTP was employed for the determination of the nucleotide sequences of these clones. DNASIS (Hitachi Soft Engineering Co. Ltd.) was used for analysis of the data. The nucleotide sequences of cDNA coding for human PACAP receptor Type I-B, Type I-B2 and  
25 Type I-C and the amino acid sequences deduced therefrom are shown in Figs. 16, 17 and 18, respectively. The nucleotide sequences of cDNA coding for human PACAP receptor Type I-B,

Type I-B2 and Type I-C are represented by SEQ ID NO:35, SEQ ID NO:36 and SEQ ID NO:37, respectively. The amino acid sequences deduced therefrom are represented by SEQ ID NO:25, SEQ ID NO:27 and SEQ ID NO:29, respectively.

5

[Example 5] Expression of Bovine PACAP Receptor Protein cDNAs

(1) Preparation of Transformants Containing Bovine PACAP Receptor Protein cDNAs

10 cDNA clone pBPR35 of the bovine PACAP receptor protein obtained in Example 2 was digested with SmaI and BamHI, thereby cutting out a fragment (about 800 bp) from the plasmid. Then, a HindIII linker was added to the SmaI-digested terminus of this fragment. The resulting fragment  
15 was named "fragment A". On the other hand, two kinds of fragments were obtained by digesting pBPR68 with BamHI and SmaI. One of them, a fragment of about 1 kbp (named "fragment B"), was cut out. These fragment A and fragment B were ligated with each other at the respective BamHI-  
20 digested sites to prepare recombinant cDNA (pBPR-T). pBPR-T was inserted in the HindIII and EcoRV sites downstream of a CMV promoter of expression vector pRc/CMV to prepare an expression vector. This expression vector was introduced into a CHO cell by the calcium phosphate method using a  
25 CellPfect transfection kit (Pharmacia) to obtain a transformant. The transformant cells were selected with 500 µg/ml G-418 (trade mark: Geneticin, Lifetech Oriental).

(2) Preparation of Membrane Fraction of the Transformants

The transformants (CHO cells) cultivated for 3 days after subculture were separated using 0.2 mM EDTA/phosphate buffer, and suspended in 10 mM sodium carbonate buffer supplemented with 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSA), 20 µg/ml leupeptin, 4 µg/ml E-64 and 1 µg/ml pepstatin. The suspended cells were disrupted with a Polytron homogenizer (Kinematica). The disrupted product was centrifuged with a high speed cooling centrifuge (CR26H, Roter RR18, Hitachi, Ltd.) at 3,000 rpm for 10 minutes to obtain a supernatant. The resulting supernatant was further ultracentrifuged with an ultracentrifuge (SCP70H, Roter RPZ35T, Hitachi, Ltd.) at 30,000 rpm for 60 minutes to obtain pellets. The resulting pellets were suspended in a buffer [20 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 2 mM EDTA, 0.5 mM PMSF, 20 µg/ml leupeptin and 1 µg/ml pepstatin] to prepare a membrane fraction suspension.

(3) Saturation Binding Experiment of Bovine PACAP Receptor Protein cDNA-Expressed CHO Cells

The membrane fraction of the transformants obtained in (2) described above was reacted with 100 pM [<sup>125</sup>I]-PACAP27 in a buffer [20 mM Tris-HCl (pH 7.4), 5 mM magnesium acetate, 2 mM EGTA, 0.5 mM PMSF, 20 µg/ml leupeptin, 4 µg/ml E-64 and 1 µg/ml pepstatin] at 25°C for 75 minutes. Bound ligands were separated from free ligands through a glass fiber filter. The non-specific binding was examined

in the presence of 1  $\mu$ M PACAP27 (Fig. 33). The binding was examined with a  $\gamma$ -ray counter. The dissociation constant and the maximum binding were examined by Scatchard plot analysis (Fig. 34).

5       (4) Competitive Binding Experiment of Bovine PACAP  
Receptor Protein cDNA-Expressed CHO Cells

Under the conditions of the binding experiment of (3) described above, PACAP27, PACAP38 and VIP were added to examine competition with [ $^{125}$ I]-PACAP27. Bovine PACAP  
10 receptor protein on the membrane fraction showed a high reactivity, but low in reactivity with VIP (Fig. 35).

(5) Assay of Intracellular Cyclic AMP Production of  
Bovine PACAP Receptor Protein cDNA-Expressed CHO Cells

Three days after the transformants (CHO cells) were  
15 plated on a 24-well plate, the cells were washed with Hank's buffer (composition: 8 g/l NaCl, 0.4 g/l KCl, 0.06 g/l  $\text{Na}_2\text{HPO}_4$ , 1.0 g/l glucose, 0.2 g/l  $\text{MgSO}_4$ , 0.14 g/l  $\text{CaCl}_2$  and 0.35 g/l  $\text{NaHCO}_3$ ) supplemented with 0.05% BSA, and treated in the presence of 0.2 mM 3-isobutyl-1-  
20 methylxanthine at 37°C for 1 hour. PACAP27, PACAP38 and VIP of various concentrations were added thereto, followed by cultivation at 37°C for 30 minutes. After the cells were washed with the above-mentioned Hank's buffer supplemented with 0.05% BSA, intracellular cyclic AMP was  
25 extracted by the use of 500  $\mu$ l of Hank's buffer and 100  $\mu$ l of 20% perchloric acid, and neutralized with 1.5 M KOH. The amount of cyclic AMP was assayed with a cAMP oxygen

immunoassay system (BIOTRAK Amersham). The concentration of intracellular cyclic AMP increased depending on the concentrations of PACAP27 and PACAP38 (Fig. 36).

(6) Assay of Intracellular Inositol Phosphate of  
5 Bovine PACAP Receptor Protein cDNA-Expressed CHO Cells

The pathway of signal transmission of inositol phosphate well known as the pathway of signal transmission together with cyclic AMP was examined. Three days after the transformants (CHO cells) were plated on a 24-well  
10 plate, 5  $\mu$ Ci myo-[ $^3$ H] inositol (19.1 Ci/mmol, Amersham) was added to the cell culture solution, followed by cultivation overnight at 37°C. The cells were washed with an assay buffer (20 mM HEPES, 140 mM NaCl, 4 mM KCl, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 1.25 mM CaCl<sub>2</sub>, 10 mM LiCl, 10 mM  
15 glucose and 0.1% BSA). Then, PACAP27, PACAP38 and VIP of various concentrations were added to 500  $\mu$ l of the assay buffer, and the mixtures were added to plates, followed by reaction with the cells at 37°C for 20 minutes. One hundred microliters of 20% perchloric acid was added  
20 thereto to stop the reaction, and intracellular inositol phosphate was extracted. The extract was neutralized with 1.5 M KOH. All inositol phosphate was separated from free inositol by the use of ion exchange chromatography (AGI-X8, Bio RAD). Thereafter, inositol phosphate was eluted with 1  
25 M ammonium formate/0.1 M formic acid, and the amount of inositol phosphate was measured with a scintillation counter. The concentration of inositol phosphate increased

depending on the concentrations of PACAP27 and PACAP38  
(Fig. 37).

[Example 6] Confirmation of Expression Site of Rat PACAP  
Receptor mRNA

(1) Preparation of Poly(A)<sup>+</sup> RNA

Total RNAs were prepared from the brains, lungs,  
livers, kidneys and testes of 8-week-old Sprague Dawley  
rats (males, Nippon Charles River) by the guanidine  
isothiocyanate method [Biochemistry, 18, 5294 (1979) and  
Method in Enzymology, 154 3 (1987)] and poly(A)<sup>+</sup> RNA  
prepared from the brains, lungs, livers, kidneys and testes  
was fractionated by formalin-modified agarose gel  
electrophoresis [Proc. Natl. Acad. Sci. U.S.A., 77, 5794  
(1980)] contained 2.2 M formalin (Wako Pure Chemical  
Industries), followed by transfer to a nylon membrane  
filter (Pole).

(2) Preparation of Probe

374-bp fragment haveing the nucleotide sequence from  
the 76th to 450th of DNA (rat PACAP receptor cDNA  
pRPACAPR12) represented by the nucleotide sequence of SEQ  
ID NO:41 was labeled with <sup>32</sup>P by the use of a multi-prime  
labeling kit (Amersham) to prepare a probe.

(3) Northern Hybridization

The filter of (1) described above was treated at 80°C  
for 2 hours to fix RNA, followed by hybridization in a



hybridization buffer [50% formamide deionized, 5 X SSPE, 5 X Denhardt's solution, 0.5% SDS, and 100 µg/ml heterologous salmon sperm DNA heat denatured after ultrasonication (Wako Pure Chemical Industries)] overnight at 42°C.

5 Subsequently, the probe obtained in (2) described above was heat denatured, and the heat-denatured probe was added thereto, followed by hybridization overnight at 42°C.

Washing was conducted 5 times with 2 X SSC, 0.1% SDS at 55°C for 30 minutes, and further twice 0.1 X SSC, 0.1% SDS at 50°C for 20 minutes. Autoradiography was carried out for 12 hours using an image analyzer (Fuji BAS-2000) to detect desired bands. Results thereof revealed that PACAP receptor mRNA was most expressed in the brains, that expression thereof was also observed in the lungs and the  
15 livers, and that the size of mRNA was about 6.5 kb (Fig. 38).

[Example 7] Expression of Rat PACAP Receptor Protein cDNA

(1) Construction of Expression Vector for Animal Cells of Rat PACAP Receptor Protein cDNA

20 Using plasmids pRPACAPR46-5 and pRPACAPR12 obtained in Example 3, an NcoI fragment having an N-terminal translation initiation codon was prepared. After repair of both ends of this fragment with Klenow fragments (Takara), HindIII linkers (Takara) were added thereto, and further  
25 cleaved with BamHI. Of the resulting fragments, a fragment containing the translation initiation codon was recovered by electrophoresis, and ligated with cDNA I obtained by

cleaving BamHI-ApoI fragments of pRPACAPR46-5 and  
pRPACAPR12 with HindIII and EcoRI, respectively, to  
construct an expression vector in which NcoI-ApoI portions  
of the respective cDNA fragments were inserted. These  
5 plasmids were further cleaved double by the use of HindIII  
and XbaI, and DNA fragments containing cDNA portions were  
incorporated into other animal cell expression vectors,  
pRc/CMV, utilizing the same sites, to obtain expression  
vectors pRPR3-A (derived from pRPACAPR46-5) and pRPR4-B  
10 (derived from pRPACAPR12).

### (2) Introduction of Expression Vector into CHO Cells

9.0 X 10<sup>5</sup> CHO cells were subcultured to each tissue  
culture flask having a bottom area of 25 cm<sup>2</sup> (Corning), and  
cultivated for 24 hours in a culture solution (culture  
15 solution A) composed of Ham's F12 medium (Flow), 10% fetal  
bovine serum, and penicillin and streptomycin as  
antibiotics. Expression vectors pRPR3-A (derived from  
pRPACAPR46-5) and pRPR4-B (derived from pRPACAPR12)  
obtained in (1) described above were introduced into CHO  
20 cells each in an amount of 10 µg with a gene introduction  
kit (CellPfect, Pharmacia) by the calcium phosphate method  
according to the formulation of the kit. After 24 hours,  
the culture solution was exchanged. After further 24  
hours, the solution was exchanged by culture solution A  
25 supplemented with 500 µg/ml of G418, and cDNA-introduced  
cells were selected, based on resistance to G418.

### (3) Binding Experiment of PACAP Receptor Protein and

[<sup>125</sup>I]-PACAP27 on CHO Cell Membrane

CHO cells exhibiting resistance to G418 were recovered by trypsin digestion, and subcultured to a 12-well plate for tissue culture. The cells were incubated until they covered the bottom surface of the tissue culture plates completely. Untreated CHO cells and rat VIP receptor cDNA-introduced CHO cells were also similarly cultivated. The cells were washed twice with a buffer for the binding experiment [Hank's solution (pH 7.4) containing 5 mM HEPES, 5% CHAPS and 0.1% BSA]. Then, the buffer and [<sup>125</sup>I]-PACAP27 were successively added so as to give a final [<sup>125</sup>I]-PACAP27 concentration of 100 pM. The amount of the reaction solution per well was 500 µl. and the radioactivity was about 11.4X10<sup>4</sup> cpm. For analysis of specificity, samples containing unlabeled PACAP27 of a final concentration of 1 µM and VIP, in addition to the samples containing only the labeled products, were prepared. After incubation at 37°C for 1 hour, the cells washed three times with the buffer for the binding experiment were dissolved with 1 ml of 0.5 N NaOH and 0.1% SDS for each well, and the radioactivity contained therein was measured with a γ-counter. Results of measurements are shown in Fig. 39. Columns 1 to 12 in Fig. 39 indicate the radioactivity in CHO cells under the following conditions:

Column 1: untreated CHO cells + [<sup>125</sup>I]-PACAP27

Column 2: untreated CHO cells + [<sup>125</sup>I]-PACAP27 + cold PACAP27

Column 3: untreated CHO cells + [ $^{125}\text{I}$ ]-PACAP27 + cold  
VIP

Column 4: pRPR3-A-introduced CHO cells + [ $^{125}\text{I}$ ]-PACAP27

Column 5: pRPR3-A-introduced CHO cells + [ $^{125}\text{I}$ ]-PACAP27

5 + cold PACAP27

Column 6: pRPR3-A-introduced CHO cells + [ $^{125}\text{I}$ ]-PACAP27  
+ cold VIP

Column 7: pRPR4-B-introduced CHO cells + [ $^{125}\text{I}$ ]-PACAP27

Column 8: pRPR4-B-introduced CHO cells + [ $^{125}\text{I}$ ]-PACAP27

10 + cold PACAP27

Column 9: pRPR4-B-introduced CHO cells + [ $^{125}\text{I}$ ]-PACAP27  
+ cold VIP

Column 10: rat VIP receptor cDNA-introduced CHO cells  
+ [ $^{125}\text{I}$ ]-PACAP27

15 Column 11: rat VIP receptor cDNA-introduced CHO cells  
+ [ $^{125}\text{I}$ ]-PACAP27 + cold PACAP27

Column 12: rat VIP receptor cDNA-introduced CHO cells  
+ [ $^{125}\text{I}$ ]-PACAP27 + cold VIP

Fig. 39 indicates that the radioactivities in the  
20 pRPR3-A-introduced CHO cells and the pRPR4-B-introduced CHO  
cells (column 4 and column 7, respectively) are higher than  
that in the untreated CHO cells (column 1). This fact  
proved that each of the pRPR3-A-introduced CHO cells and  
the pRPR4-B-introduced CHO cells produced PACAP receptors.

25 (4) Analysis of Specificity of Rat PACAP Receptor on  
CHO Cell Membrane Using [ $^{125}\text{I}$ ]-PACAP27

The pRPR3-A-introduced and pRPR4-B-introduced CHO

cells obtained in (2) described above were each disrupted in sodium carbonate buffer containing 1 mM EDTA, 0.5 mM PMSF, 20 µg/ml leupeptin, 4 µg/ml E-64 and 1 µg/ml pepstatin with a Polytron homogenizer (Kinematica) to

5 prepare membrane fractions. Using the membrane fractions, complex binding experiments were conducted. For each of the membrane fractions of the pRPR3-A-introduced CHO cells and the pRPR4-B-introduced CHO cells, each of 10 µg and 15 µg (converted to a protein amount) thereof was ligated with

10 100 pM of [<sup>125</sup>I]-PACAP27 in a buffer containing 20 mM Tris (pH 7.4), 1 mM EDTA, 0.05% CHAPS, 0.1% BSA and various protease inhibitors. For the competitive experiments, PACAP27 and VIP having each concentration were added. The reaction was conducted at 25°C for 1 hour, and bound

15 ligands were separated from free ligands by filtration through a filter. As to non-specific binding, a value in the case that 1 µM unlabeled PACAP27 was added and used as a standard. The amount of bound ligands was measured with a γ-counter. After elimination of the non-specific

20 binding, it was examined whether or not concentration-dependent competition took place. Results thereof revealed that concentration-dependent competition took place. For VIP similar to PACAP27 in structure, competition was observed only at a concentration much higher than that of

25 PACAP27, which showed that the PACAP receptor protein which was allowed to express was PACAP-specific (Fig. 40).

#### (5) Screening of Clones Highly Producing Rat PACAP

## Receptor Protein by Binding Experiment with [ $^{125}$ I]-PACAP27

The rat PACAP receptor protein cDNA-introduced CHO cells obtained in (2) described above were each subcultured to 10-cm diameter dishes at a low density. After  
5 cultivated until formation of colonies, each of the colonies was dispersed and recovered by suction. Cells derived from the respective colonies were separately subcultured in 6-well plates for tissue culture, followed by binding experiments using parts thereof in a manner  
10 similar to that of (4) described above (Fig. 41). Clones having relatively more bound [ $^{125}$ I]-PACAP27 when compared among wells were selected, and the reproducibility was further confirmed. As a result, clones A12 and B17 reproducibly binding to [ $^{125}$ I]-PACAP27 much more were  
15 selected from the pRPR3-A-introduced pRPR4-B-introduced CHO cells (Fig. 42).

## (6) Assay of Intracellular Cyclic AMP of Rat PACAP Receptor Protein cDNA-Introduced CHO Cells

From the binding experiment with [ $^{125}$ I]-PACAP27 of (3)  
20 described above, using CHO strains A12 and B17 highly producing rat PACAP receptor protein, the production promotion of intracellular cyclic AMP with PACAPs was detected in the following manner. Each 48-well plate for tissue culture was inoculated with each of the cell strains  
25 at a density of  $1.0 \times 10^5$  cells/well, followed by cultivation for 3 days. The plate was washed twice with Ham's F12 medium supplemented with 0.1% BSA and 0.5 mM IBMX, and 500

μl/well of the same medium was added thereto. PACAP27, PACAP38 or VIP having each concentration was added thereto in a 1/100 amount, followed by standing at 37°C for 40 minutes. The supernatant was removed, and extraction was conducted with 100% cold ethanol. The extract was evaporated to dryness with a centrifugal freeze dryer, and redissolved in the buffer attached to an EIA kit for assaying cyclic AMP (Amersham). Then, the amount of cyclic AMP was assayed according to the formulation of the kit.

10 Results thereof revealed that both A12 and B17 promoted the production of intracellular cyclic AMP, depending on the concentrations, for PACAP27 and PACAP38, but a concentration much higher than that of the PACAPs was required to promote the production of intracellular cyclic

15 AMP, for VIP (Fig. 43).

#### (7) Construction of Rat PACAP Receptor Protein cDNA Expression System Using Baculovirus

Animal cell expression vectors pRPR3-A and pRPR4-B were each cleaved with HindIII, and the termini were repaired with Klenow fragments (Takara), followed by addition of BglII linkers. The resulting fragments were further cleaved with XbaI, and the termini were repaired with Klenow fragments, followed by addition of HindIII linkers. These DNAs were each digested double by the use of HindIII and BglII, thereby obtaining DNA fragments corresponding to translation regions. pBlneBacIII, a baculovirus transfer vector, was similarly digested double

with HindIII and BglII, and subjected to ligation reaction with the above-mentioned DNA fragments. According to the formulation of a kit (Maxbac baculovirus expression system, Invitrogen) with which plasmid DNA confirmed in insertion  
5 was prepared, the resulting fragments, together with baculovirus genome DNA, were introduced into Sf9 cells. After cultivation at 27°C for 2 days, virus particles appeared in the supernatant were recovered. Recombinant viruses were selected therefrom by the plaque assay in  
10 accordance with the formulation of the kit.

(8) Expression Using Rat PACAP Receptor Protein cDNA-  
Introduced Baculovirus

The recombinant plaques formed by the plaque assay were extracted with a micropipette, and dispersed in 1 ml  
15 of complete medium for Sf9 [Grace medium for insects (Gibco) containing necessary additives, inactivated calf serum and gentamicin]. A 25-cm<sup>2</sup> flask for tissue culture was inoculated with 2X10<sup>6</sup> Sf9 cells, together with 5 ml of the medium, and the cells were adhered to a bottom of the  
20 flask, followed by addition of 500 µl of the above-mentioned virus solution. After cultivation at 27°C for 5 days, the cells were recovered by pipetting. The cells were pelletized by centrifugation, and suspended in a small amount of medium. Then, a 1/10 amount of the suspension  
25 was poured into each Eppendorf tube. After further centrifugation, the supernatant was replaced by the same buffer as with the binding experiment in animal cells



(composition: Hank's solution (pH 7.4) containing 5 mM HEPES, 5% CHAPS and 0.1% BSA]. Then, [ $^{125}$ I]-PACAP27 was added so as to give a final concentration of 100 pM, and unlabeled PACAP27 of a final concentration of 1  $\mu$ M was added to a sample for analysis of specificity to make up a total solution amount of 500  $\mu$ l. After standing at room temperature for 1 hour and binding, bound ligands, together with the cells, were pelletized by centrifugation. The pellets were further resuspended in the same buffer and centrifuged. After this procedure was repeated three times to conduct sufficient washing, the amount of radioligands remaining in the pellets was measured with a  $\gamma$ -counter. As a result, 4 virus clones showing a very high binding were obtained (Fig. 44).

15 [Example 8] Expression of Human PACAP Receptor Protein cDNA

(1) Preparation of Transformant in Baculovirus System Using Human PACAP Receptor Protein cDNA

A fragment cut out by digestion with BamHI and PstI from animal cell expression vector pCDNAI/Amp in which human PACAP receptor protein was subcloned was inserted in the BamHI and PstI sites of transfer vector pBlueBacIII to prepare a recombinant transfer vector. Sf9 cells were transfected with this vector, together with baculovirus DNA (AcMAPV DNA), using the transfection module attached to the kit (MAXBAC, Inbitrogen). After transfection, the viruses appeared in the supernatant, so that the culture supernatant of the fourth day was used as a virus solution.

Sf9 cells ( $2 \times 10^6$  cells) seeded on a 6-cm<sup>2</sup> dish were infected with this virus solution at room temperature for 30 minutes, and a medium containing 0.6% agarose was poured therein for fixing. Cell culture at a high humidity (a  
5 humidity of 100%) for 5 to 6 days resulted in development of virus plaques. Plaques caused by viruses in which human PACAP receptor protein was recombined could be judged by turning blue, and the viruses were recovered. The recombinants were purified by repetition of this plaque  
10 assay. Sf9 cells were infected with the purified recombinants, and cultivated for 48 to 72 hours, whereby PACAP receptor protein-expressed transformants could be obtained (Fig. 45).

## (2) Construction of Cell Strain Expressing Human PACAP 15 Receptor

An overall length fragment was cut out from human PACAP receptor cDNA-cloned pTS847-1 by digestion with EcoRI, and inserted in the EcoRI site of animal cell expression vector pRc/CMV so as to be arranged in a correct  
20 direction, thereby constructing pTS849. The resulting plasmids were introduced into CHO-K1 cells (ICN) by the calcium phosphate method, and plasmid-incorporated clones were selected with 500 µg/ml G-418 (Geneticin).

## (3) Scatchard Plot Analysis Using Membrane Fraction of 25 Human PACAP Receptor Protein-Expressed CHO-K1 Cells and Competitive Inhibition Analysis

The human PACAP receptor-expressed CHO-K1 cells

obtained in (2) described above were cultivated in ten 175-  
cm<sup>2</sup> flasks containing a medium supplemented with 500 µg/ml  
G-418 (trade mark: Geneticin, Lifetech Oriental). When the  
cells covered almost entire bottom surfaces of the flasks,  
5 the CHO-K1 cells were separated with PBS solution  
containing 1 mM EDTA. After washing with the same buffer,  
the CHO-K1 cells were suspended in 10 mM NaCO<sub>3</sub> buffer  
containing 1 mM EDTA, 0.5 mM PMSF, 20 µg/ml leupeptin, 20  
µg/ml E-64 and 1 µg/ml pepstatin, and disrupted with a  
10 Polytron homogenizer (Kinematica). Then, the disrupted  
product was centrifuged with a high speed cooling  
centrifuge (CR26H, Roter RR18, Hitachi, Ltd.) at 3,000 rpm  
for 10 minutes. The resulting supernatant was further  
ultracentrifuged with an ultracentrifuge (SCP70H, Roter  
15 RP42, Hitachi, Ltd.) at 30,000 rpm for 60 minutes. The  
resulting pellets were suspended in a buffer containing 20  
mM Tris-HCl (pH 7.4), 0.25 M sucrose, 2 mM EDTA, 0.5 mM  
PMSF, 20 µg/ml leupeptin and 1 µg/ml pepstatin. The  
resulting suspension was used as a membrane fraction.

20 The preparation of [<sup>125</sup>I]-PACAP27, the Scatchard plot  
analysis obtained from the saturation binding experiment,  
and the competitive inhibition experiment were carried out  
in accordance with the method described in Example 1 (3).

From results of Scatchard plot analysis, a single  
25 binding site existed in the membrane fraction of the human  
PACAP receptor protein-expressed CHO-K1 cells, and the  
dissociation constant (K<sub>d</sub>) was 41±6.9 pM (Fig. 46).

Further, results of the competitive inhibition experiment proved that PACAP27 and PACAP38 competed with [ $^{125}$ I]-PACAP27. On the other hand, it was revealed that VIP was 1,000 times weaker than PACAP27 (Fig. 47).

5        (4) Assay of Intracellular Cyclic AMP of Human PACAP Receptor Protein-Expressed CHO-K1 Cells

TE330463600  
The human PACAP receptor protein-expressed CHO-K1 cells obtained in (2) described above were cultivated in a 24-well plate containing a medium supplemented with 500  
10  $\mu$ g/ml G-418 (trade mark: Geneticin, Lifetech Oriental) until the cells almost covered an entire surface of the plate. After washing twice with Hank's buffer containing the culture buffer, 10 mM HEPES and 0.05% BSA, the CHO-K1 cells were cultivated in the above-mentioned buffer  
15 supplemented with 0.2 mM 3-isobutyl-1-methylxanthine at 37°C for 60 minutes. Then, PACAP27, PACAP38 or VIP having each concentration was added thereto, followed by further cultivation at 37°C for 30 minutes. After absorption of the buffer, the cells were washed twice with the culture  
20 buffer. Then, cAMP was extracted from the cells with 20% perchloric acid. After transfer to a 1.5-ml Eppendorf tube, the extract was centrifuged with a Tomy microcentrifuge at 12,000 rpm for 5 minutes, and the supernatant was neutralized with 1.5 N KOH/60 mM HEPES to  
25 prepare a cell eluted solution. The concentration of cyclic AMP was determined by the acetylation method of a cAMP assay system (Amersham). Under these determination

conditions, when nothing was added, the amount of intracellular cAMP was 0.7 pmole/well. For PACAP27 and PACAP38, the concentration of intracellular cAMP increased depending on the concentrations. In particular, when 0.1 mM of PACAP38 was added, accumulation of cyclic AMP about 30 times the basal level (about 21 pmoles/well) was observed (Fig. 48). VIP little raised the concentration of intracellular cyclic AMP, compared with PACAP27 and PACAP38 (Fig. 48).

10 [Example 9] Expression of Human PACAP Receptor mRNA

Poly(A)<sup>+</sup> RNA (Clontech) from each human tissue was subjected to 1.1% agarose gel-modified gel electrophoresis containing 2.2 M formalin for fractionation, followed by transfer to a nylon membrane filter. Then, RNA transferred was fixed to the nylon membrane with UV. A probe of human PACAP receptor cDNA (SacI-BglII fragment of pTS847-1, nucleotide No. 168-562) was prepared with a random prime labeling kit (Amersham) and [ $\alpha$ -<sup>32</sup>P]dCTP (Du Pont/NEN), and northern hybridization was carried out using this probe.

20 As a result, human PACAP mRNA was most expressed in the brain, and the size thereof was about 7 kb. Expression was also observed in the lung, the liver, the pancreas and other organs, although weak (Fig. 49).

[Example 10] Expression of PACAP mRNA in Rat Central Nerve

25 System

All RNAs were prepared from the olfactory bulbs, amygdalae, cerebral basal ganglia, hippocampi, thalami,

hypothalami, cerebral cortices, medulla oblongatas, cerebellums, spinal cords and pituitary glands of 8-week-old S. D. rats ( $\delta$ ) by the guanidine isothiocyanate method, and poly(A)<sup>+</sup> RNA was further prepared by the use of an oligo(dT) spun-column (Pharmacia). Five micrograms of poly(A)<sup>+</sup> RNA prepared from the above regions of the central nervous system was fractionated by 1.2% formalin-modified agarose gel electrophoresis [Proc. Natl. Acad. Sci. U.S.A., 77, 5794 (1980)] contained 2.2 M formalin (Wako Pure Chemical Industries), followed by transfer to a nylon membrane filter (Pole).

## (2) Preparation of Probe

374-bp fragment having the nucleotide sequence from the 76th to 450th of DNA (rat PACAP receptor cDNA pRPACAPR12) represented by the nucleotide sequence of SEQ ID NO: 41 was labeled with <sup>32</sup>P by the use of a multi-prime labeling kit (Amersham) to prepare a probe.

## (3) Northern Hybridization

The filter of (1) described above was treated at 80°C for 2 hours to fix RNA, followed by hybridization in a hybridization buffer [50% formamide deionized, 5 X SSPE, 5 X Denhardt's solution, 0.5% SDS, and 100 µg/ml heterologous salmon sperm DNA heat denatured after ultrasonication (Wako Pure Chemical Industries)] overnight at 42°C. Subsequently, the probe obtained in (2) described above was heat denatured, and the heat-denatured probe was added thereto, followed by hybridization overnight at 42°C.

Washing was conducted 5 times with 2 X SSC, 0.1% SDS at room temperature for 5 minutes, and further twice 0.1 X SSC, 0.1% SDS at 50°C for 20 minutes. Autoradiography was carried out for 7 days using a X-OMAT AR film (Kodak) to detect desired bands.

Results thereof revealed that rat PACAP receptor mRNA was expressed in almost all regions of the central nerve system, and that there was little expression in the cerebellums and pituitary glands (Fig. 50). From these results, the PACAPs are deduced to play an important role in the central nerve system.

[Example 11] Screening of Human PACAP Receptor Antagonist Which Uses Cell Membrane Fraction of Sf9 cell Expressing cDNA of Human PACAP Receptor Protein

(1) Preparation of Buffer for Assay

Composition of buffer

20mM Tris-HCl, 2mM EGTA, 5mM  $(\text{CH}_3\text{COO})_2\text{Mg} \cdot 4\text{H}_2\text{O}$ , 0.5mM PMSF, 1  $\mu\text{g/ml}$  pepstatin, 20  $\mu\text{g/ml}$  leupeptin, 4  $\mu\text{g/ml}$  E-64, 0.03%  $\text{NaN}_3$ , 0.1% BSA, 0.05% CHAPS, pH7.2

Method for preparation

The agents other than peptidase inhibitor (PMSF, pepstatin, leupeptin, E-64) and BSA were dissolved into distilled water. While controlling pH of the aqueous solution with 6N HCl, peptidase inhibitor was added thereto. Pepstatin and PMSF were dissolved into DMSO and the DMSO solution was added to the distilled water solution with rapid agitation. Final concentration of DMSO was

adjusted to 0.1%, thus pepstatin and PMSF were dissolved into 1ml of DMSO to prepare 1 liter of buffer. Then the solution was mixed and BSA was added thereto.

(2) Sf9 cells which express human PACAP receptor protein obtained in Example 8 were disrupted by Polytron mixer in a buffer for homogenize (20 mM Tris-HCl, 2mM EDTA, 0.5mM PMSF, 1 µg/ml pepstatin, 20 µg/ml leupeptin, 4 µg/ml E-64, pH7.4). The disrupted cell solution was centrifuged at 3,000 rpm for 5 minutes and the supernatant was centrifuged at 30,000 rpm for 60 minutes. The resulting precipitate was treated as a membrane fraction. The membrane fraction was diluted with the buffer for assay to 2 µg protein/ml. The diluted solution was applied on a cell strainer (FALCON, 2350) and was divided into 100 µl in each tube (FALCON, 2053) with dispenser.

(3) Each 1 µl of 10 mM of the sample was added to the reaction tubes (final concentration: 100 µM, room temperature). DMF was added thereto for assay of the maximum binding amount, and 1 µl of DMF with 100 µM PACAP27 was added for assay of nonspecific binding amount (final concentration: 1 µM). The maximum binding amount was assayed twice respectively at the beginning and the end of the assay, and the nonspecific binding amount was assayed twice at the end of the assay.

(4) In radio isotope region, each 2 µl of 5 nM [<sup>125</sup>I]-PACAP27 (DuPont) was added in the reaction tubes (final concentration: 100 pM). [<sup>125</sup>I]-PACAP27 was placed on ice.



(5) The reaction tubes were incubated at 25°C for 1 hour.

(6) 1.5 ml of a detergent buffer was added into the reaction tubes and the mixture was filtered on a glass fiber paper (Whatman, GF/F) using Sampling manifold

5 (Millipore). 1.5 ml of a detergent buffer was further added to the reaction tubes and they were filtered. The glass fiber filtration paper (Whatman, GF/F) was previously immersed in a PEI (polyethyleneimine) solution (20 mM Tris-HCl, 0.3% PEI, pH7.4). The detergent buffer may be similar  
10 with the assay buffer but it is not necessary to contain peptidase inhibitor.

(7) [<sup>125</sup>I] remaining on the glass fiber filtration paper was counted by γ-counter. Based on the counts, inhibiting activity on a binding specificity [Percent Maximum Binding]  
15 of PACAP27 and a PACAP27 receptor of the samples were determined according to the following formula:

$$PMB = [(B - NSB) / B_0 - NSB] \times 100$$

PMB: percent Maximum Binding

B : value when the samples are added,

20 NSB: non-specific binding amount

B<sub>0</sub> : Maximum Binding

As a result, substances No.1 to 10 as shown in Fig.51 were obtained as substances which inhibited a specific binding of PACAP27 and PACAP receptor. PMB of the  
25 compounds are shown in Table 3.

Table 3

Test compound No.	Specific Binding %
1	57
2	11
3	37
4	15
5	2
6	-3
7	50
8	15
9	20
10	34

[Example 12] Preparation of Anti-PACAP Receptor Antibody

(1) Preparation of a Partial Peptide of PACAP Receptor

The 5th Cys(C) of the amino acid sequence, MHSDCIFKKKEQAMC, was substituted with Ala(A) for the convenience of a preparation of immunoantigen complexes to obtain a partial peptide, MHSDAIFKKKEQAMC, with a conventional method using a autosynthesizer (430A, AppliedBiosystem). The first amino acid sequence corresponds to 1st to 14th amino acid sequence of SEQ ID NO:14, which is a common sequence to bovine, rat or human PACAP receptor represented by the amino acid sequence of anyone of SEQ ID NO:14 to SEQ ID NO:29.

(2) Preparation of Immunogen

A complex of the synthetic peptide (MHSDAIFKKEQAMC) obtained in the above (1) and bovine thyroglobulin (BTG) was made and used as an immunogen. Thus, 21 mg of BTG was dissolved into 1.4 ml of 100 mM phosphate buffer (pH 6.8) and the solution was mixed with 2.35 mg of GMBS in 100  $\mu$ l of DMF to react at room temperature for 40 minutes. The reactant was applied on Sephadex G-25 column<sup>TM</sup> (1x35 cm) equilibrated with 100 mM phosphate buffer and to obtain a fraction containing BTG. A half (1.5 ml) of the fraction was mixed with 2 mg of the synthesized peptide dissolved in 50% DMSO to react at 4°C for two days. The reactant was dialyzed against physiological saline at 4°C for two days and the dialyzate was divided into small amount and freeze-restored.

(3) Immunization

100  $\mu$ g of the immunogen obtained in the above (2) was subcutaneously given with a complete Freund's adjuvant to each female BALB/c mouse of 6 to 8 week old. Once or twice additional immunizations was conducted at three weeks intervals.

(4) Preparation of HRP-labelled partial peptide of receptor

HRP (Horse radish peroxidase)-labelled partial peptide necessary for assay for antibody value with EIA was prepared as follows:

Twenty(20) mg of HRP was dissolved into 1.5 ml of phosphate buffer (pH6.5) and the solution was mixed with

1.4 mg of GMBS [N-(6-maleimidebutyloxy)succinimide] in 100  $\mu$ l of DMF to react at room temperature for 40 minutes. The reactant was applied on Sephadex G-25 column<sup>TM</sup> (1x35 cm) equilibrated with 100 mM phosphate buffer and to  
5 obtain a fraction containing BTG. A half (1.5 ml) of the fraction was mixed with 2 mg of the synthesized peptide dissolved in 50% DMSO to react at 4°C for two days. The reactant was applied on Ultrogel ACA44 column<sup>TM</sup> (1x35 cm) equilibrated with 100 mM phosphate buffer and to obtain a  
10 fraction containing HRP-labelled partial peptide. BSA (final concentration: 0.1%) and thimerosal (final concentration: 0.05%) were added to the fraction to be restored at 4°C.

#### (5) Assay of Antibody Titer

15 Antibody titer of antiserum of the mice immunized in the above (3) was assayed as follows:

100  $\mu$ l of 100  $\mu$ g/ml anti-mouse immuno globulin antibody (IgG fraction, Cappel) dissolved in 100 mM carbonate buffer (pH 9.6) was added to a 96-well plate and  
20 kept at 4°C for 24 hours to make an anti-mouse immunoglobulin bound microplate. After the plate was washed with phosphate bufferized physiological saline (PBS, pH 7.4), 300  $\mu$ l of Blockace (Yukizirushi, Japan) diluted to 25% with PBS was added to the plate to react at 4°C for at  
25 least 24 hours in order to block the remaining binding sites of the plate.

50  $\mu$ l of Buffer A (0.1% BSA, 0.1M NaCl, 1mM MgCl<sub>2</sub>,

0.05% CHAPS and 0.1%  $\text{NaN}_3$  in 20mM phosphate buffer, pH 7.0) and 100  $\mu\text{l}$  of mouse anti-partial peptide of PACAP receptor-antiserum diluted with Buffer A were added to each of the well of the above anti-mouse immunoglobulin bound

5 microplate and to react at  $4^\circ\text{C}$  for 16 hours. After the plate was washed with PBS, 100  $\mu\text{l}$  of HRP-labelled peptide diluted to 300 times with Buffer B (0.1% BSA, 0.4M NaCl and 2 mM EDTA in 20mM phosphate buffer, pH 7.0) was added to react at  $4^\circ\text{C}$  for 7 hours. Then, the plate was washed with

10 PBS and 100  $\mu\text{l}$  of TMB microwell peroxidase substrate system (Kirkegaard & Perry Lab, Inc.) was added to each well to react them at room temperature for 10 minutes. 100  $\mu\text{l}$  of 1M phosphoric acid was added to each well to stop the reaction and their absorptions at 450 nm was assayed with a

15 plate reader (MTP-120, Corona).

#### (6) Preparation of Anti-partial Peptide of PACAP Receptor Monoclonal Antibody

On mice which show relatively high antibody value,

20 final immunization by intravenous injection of 200 to 300  $\mu\text{g}$  of immunogen in 0.25 to 0.3 ml of physiological saline was conducted. Spleens were enucleated from the mice after 3 to 4 days of the final immunization and pressed and filtered through a stainless mesh and the filtrate was

25 suspended in Eagle's Minimum Essential Medium (MEM) to obtain a spleen cell suspension. Mieloma cell P3-X63.Ag8.U1(P3U1 cell) derived from BALB/c mouse was used as

a cell for cell fusion [Current Topics in Microbiology and Immunology, 81, 1(1978)]. The cell fusion was conducted according to the original method. Spleen cells and P3U1 cells were respectively washed 3 times with MEM having no serum, and then they were mixed at 5:1 in the ratio of spleen cells to P3U1 cells followed by centrifugation at 700 rpm for 15 minutes to make the cells precipitate.

After thoroughly removing the supernatant, the precipitate was softly mixed and 0.4 ml of 45% polyethyleneglicol (PEG)

6000 (Kochlight) was added thereto and the mixture was maintained in water bath at 37°C for 7 minutes for the hybridization. 15 ml of MEM was slowly added by 2 ml per minute thereto and the mixture was centrifuged at 750 rpm for 15 minutes to obtain the cell precipitate. The cells were suspended mildly into 200 ml of GIT medium containing 10% fetal calf serum (Wako Pure Chemical Industry, Japan) (GIT- 10% FCS) and a 24 well multidish(Limbro) was seeded with 1 ml of the suspension to each well and incubated in an incubator with 5% carbonic acid at 37°C. After 24 hours of the incubation, 1 ml of GIT- 10% FCS containing HAT (0.1mM hypoxanthine, 0.4 µM aminopterin, 1.6 mM thymidine) (HAT medium) was added to each well and HAT selective cultivation began. After 4 and 8 days from the beginning of the cultivation, 1 ml of the culture solution was changed with new HAT medium. Growth of hybridoma was found after 8 to 10 days from the cell fusion and the supernatant when the culture solution changed yellow was taken and

assayed according to the method described in Example 5.

Typical screening of hybridoma derived from mice immunized with a partial peptide of PACAP receptoris shown in Fig. 52. There are a few colonies of hybridoma in the 5 wells and 3 wells were chosen, cloning of an antibody producing strain with limiting dilution analysis was conducted to obtain three hybridomas which produce anti-partial peptide of PACAP receptor (PRN1-25, PRN1-109 and PRN1-159). As a feeder cell for the cloning, thymus cells 10 of BALB/c mouse was employed. One(1) to three(3) x 10<sup>6</sup> cells of these hybridomas were intraabdominally administered to BALB/c mice to which 0.5 ml of mineral oil was intrabdominally administered, and 10 to 15 days after the administration, ascites containing antibodies was 15 collected.

Monoclonal antibodies were purified from the obtained ascites using a column to which Protein A was fixed. Thus, the ascites was diluted with equivalent binding buffer (3.5M NaCl, 0.05% NaN<sub>3</sub> in 1.5 M glycine, pH 9.0) and the 20 dilution was applied on Recombinant Protein A-agarose (Repligen) equilibrated with the binding buffer, washed with the buffer and antibodies were eluted with an elution buffer (0.1 M citrate buffer with 0.05% NaN<sub>3</sub>, pH 3.0). The purified monoclonal antibodies eluted were dialyzed against 25 PBS containing 0.05% NaN<sub>3</sub> at 4°C for two days and the dialysate was restored at 4°C. The monoclonal antibodies obtained are shown in Table 4.

Table 4

	Monoclonal antibodies	Type
	PRN1-25a	IgG1
5	PRN1-109a	IgG1
	PRN1-159a	IgG1

(7) Detection of PACAP Receptor by Western Blotting with  
Anti-partial Peptide of PACAP Receptor Antibody

10 Human PACAP receptor was expressed in an insect cell  
using Baculo virus and a membrane fraction was prepared  
from the cell. Membrane protein was solubilized with  
digitonin from the membrane fraction and concentrated on  
DEAE-Toyopearl column. The concentrated membrane protein  
15 solution was isolated with SDS-polyacrylamide  
electrophoresis and transferred to PVDF membrane (Applied  
Biosystem). The PVDF membrane transferred with protein was  
immersed in 5% BSA solution at 37°C for 1 hour to saturate  
adsorption sites. The PVDF membrane was washed and  
20 immersed in 10 µg/ml PRN1-159a antibody solution at room  
temperature for 1 hour. After washing, the membrane was  
immersed at room temperature for 2 hour in a solution with  
golden-colloid-labelled anti-mouse IgG and anti-mouse IgM  
antibodies (Amershum, Auroprobe BL plus GAM IgG+IgM).  
25 After washing, the membrane was treated with a sensitizer  
(Amershum, Intense BL silver enhancement Kit) and a band of  
PACAP receptor which was recognized with the antibodies was



detected (Fig. 53).

(8) Inhibition of PACAP binding by monoclonal antibodies

Membrane protein was solubilized with digitonin from the bovine brain membrane fraction and concentrated on

5 DEAE-Toyopearl column. The concentrated membrane protein solution was diluted with assay buffer (20 mM Tris, 5 mM magnesium acetate, 2mM EGTA, 0.1% BSA, 0.05% digitonin, 0.03%  $\text{NaN}_3$ , 0.5 mM PMSF, 20  $\mu\text{g/ml}$  leupeptin, 4  $\mu\text{g/ml}$  E-64, 1  $\mu\text{g/ml}$  pepstatin, pH 7.2) to 90  $\mu\text{l}$  and was added with 10  
10  $\mu\text{l}$  of the purified monoclonal antibody solution. After mixing, the solution was kept at 4°C for 16 hours and 2  $\mu\text{l}$  of 5 nM radioactive iodine-labelled PACAP27 ( $[^{125}\text{I}]\text{PACAP27}$ ) solution was added thereto to react at 25°C for 1 hour.

After completion of incubation, 1.5 ml of assay buffer  
15 detergent (digitonin in assay buffer was substituted with CHAPS of equivalent concentration) was added to the reaction solution and then the solution was filtered on a glass-fiber filter paper which was previously treated with 0.3% polyethyleneimine. The filter paper was further  
20 washed with equivalent amount of assay buffer detergent and the captured radio-activity was counted and radio-active PACAP27 bound to the receptor was determined. As shown in Fig. 54, PRN1-159a inhibited binding of  $[^{125}\text{I}]\text{PACAP27}$  to the receptor.

25 It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light

thereof will be suggested to persons skilled and purview of this Application and the scope of the appended claims.